Molecular Mechanisms of Immunometabolic Dysfunction in Multiple Sclerosis

DISSERTATION

zur Erlangung des akademischen Grades
Doctor rerum naturalium (Dr. rer. nat.)

eingereicht an der
Lebenswissenschaftlichen Fakultät der Humboldt-Universität zu Berlin

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Tag der mündlichen Prüfung: Berlin, 04. September 2019

CHARITÉ
UNIVERSITÄTSMEDIZIN BERLIN

HUMBOLDT-UNIVERSITÄT ZU BERLIN

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"According to Darwin's Origin of Species,
it is not the most intellectual of the species that survives;
it is not the strongest that survives;
but the species that survives is the one
that is able best to adapt and adjust
to the changing environment
in which it finds itself."
Leon C. Megginson paraphrasing
Charles Darwin's *Origin of Species*

Abstract

Multiple Sclerosis (MS) is a chronic neurodegenerative disease of the central nervous system characterized by autoimmune-mediated mechanisms. T cells have been associated as central pro-inflammatory mediators in MS pathogenesis. In healthy individuals, immune cells adapt metabolic programs like mitochondrial respiration and glycolysis based on their function and inflammatory phenotype. However, the relevance of metabolic reprogramming and associated pro-inflammatory mechanisms in T cell subpopulations in MS disease is not well understood yet. To address this question, Relapsing Remitting MS (RRMS) patients and meticulously matched healthy control (HC) participants were recruited as part of the clinical study *Depression and Immune Function in MS* (n=62). Blood samples, after a period of fasting, were collected and $CD4^+$ and $CD8^+$ T cells isolated from peripheral blood mononuclear cells (PBMC). The results obtained demonstrated decreased mitochondrial and glycolytic activity specific to $CD4^+$ T cells in the MS patient cohort compared to the HC participant cohort. Furthermore, increased CPT1a mitochondrial membrane protein levels were detected in $CD4^+$ T cell subpopulations in the MS patient cohort as assessed in comprehensive flow cytometry PBMC phenotype investigations. The analysis of the $CD4^+$ $CD25^-$ $CD127^+$ conventional T cell subpopulation moreover revealed a trend of decreased IL7-Rα expression levels in MS patients. Gene expression measurements of pro-inflammatory and metabolic genes did not reveal alterations in MS patients' T cell subpopulations. The results obtained in this study allude to dysfunctions in metabolic reprogramming in T cell subpopulations in MS patients and help to better understand the contribution of immunometabolism in the pathogenesis of MS disease.

Zusammenfassung

Multiple Sklerose (MS) ist eine chronische neuro-degenerative Erkrankung des zentralen Nervensystems, die durch auto-immun-bedingte Prozesse charakterisiert ist. T Zellen wurden als wesentliche pro-inflammatorische Mediatoren mit der Pathogenese der MS assoziiert. In gesunden Individuen passen Immunzellen ihren Metabolismus, wie die mitochondriale Atmung und Glykolyse, ihrer jeweiligen Funktion und ihrem inflammatorischen Phänotyp an. Im Krankheitsverlauf der MS ist die Bedeutung der metabolischen Anpassung und der damit verbundenen pro-inflammatorischen Mechanismen von T Zell-Subpopulationen noch nicht eindringlich erforscht. Um dieser Fragestellung nachzugehen wurden Relapsing Remitting MS (schubförmig, RRMS) Patienten und sorgfältig aufeinander abgestimmte gesunde Kontrollprobanden als Teil der Studie *Depression und Immunfuktion bei MS* rekrutiert (n=62). Den Patienten und gesunden Kontrollprobanden wurde Nüchternblut entnommen, woraus periphäre mononukleäre Blutzellen (PBMC) aufgearbeitet wurden, um anschließend $CD4^+$ und $CD8^+$ T Zellen zu isolieren. Die erzielten Ergebnisse zeigten $CD4^+$ T Zell-spezifische Verringerungen der mitochondrialen Atmung und glykolytischen Aktivität in der MS Patienten Kohorte im Vergleich zur Kohorte der gesunden Kontrollprobanden. Darüberhinaus wurden, zusätzlich zu den umfangreichen phänotypischen Charakterisierungen der PBMCs via Durchflußzytometrie, erhöhte Werte des mitochondrialen Membranproteins CPT1a in $CD4^+$ T Zell-Subpopulationen in der MS Patienten Kohorte detektiert. Die Analyse der $CD4^+$ $CD25^-$ $CD127^+$ konventionellen T Zell-Subpopulation ergab leicht erniedrigte Werte von IL7-Rα in MS Patienten. Genexpressionsanalysen, die mit pro-inflammatorischen und metabolischen Genen assoziiert sind, ergaben keine Veränderungen in den T Zell-Subpopulationen der MS Patienten. Die in dieser Studie erzielten Ergebnisse weisen auf Funktionsstörungen bei der metabolischen Anpassung in T-Zell-Subpopulationen bei MS Patienten hin und helfen, den Beitrag des Immunmetabolismus bei der Pathogenese der MS Erkrankung besser zu verstehen.

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1 Introduction

1.1 Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic neurodegenerative disease of the central nervous system (CNS) characterized by the demyelination of neurons believed to be induced by autoimmune mechanisms. Worldwide, approximately 2.5 million people are diagnosed with MS and about 200 000 people in Germany.

Disease influencing factors include environmental and genetic components leading to a broad and heterogeneous clinical presentation. To date, magnetic resonance imaging (MRI) as well as cerebral spinal fluid (CSF) analyses provide the basis for the clinical diagnosis, characterization and progression of MS and contribute to the understanding of the disease.

1.1.1 Disease Progression, Symptoms and Risk Factors

MS is characterized by a chronic state of neuroinflammation of the CNS, which progresses over time. The average age of disease onset is 30 years and due to increasing physical impairments, considering current treatment, approximately 50% of patients require a wheelchair 25 years after diagnosis [1]. The course of disease and disability progression depends on the kind of MS.

Disease development can generally be characterized by three stages: 1) a pre-clinical, 2) a relapsing-remitting MS (RRMS) and 3) a progressive stage. Figure 1 shows the course of disease with an overall increasing disease burden including clinical disability and MRI activity as well as decreasing brain volume over time. The pre-clinical stage oftentimes goes unrecognized unless MRI scans incidentally discover typical neuronal inflammatory regions, defined as brain lesions. This stage of MS is mostly asymptomatic and defined as the radiologically isolated syndrome (RIS). Following the RIS, usually the clinically isolated syndrome (CIS) manifests itself and is followed by the RRMS stage. Now, in CIS and RRMS, patients show distinct clinical symptoms, most prominently optical neuritis, sensory deficits and motor dysfunctions. As relapses increase over time, symptoms accumulate and disability worsens. In the RRMS stage, relapses with neurological dysfunction and

neuronal inflammation are redeemed by phases of remission during which symptoms decline or, especially in the early phases, subside to a great extent. However, with accumulating relapses, the disease burden increases, brain volume decreases and patients enter the third clinical stage, progressive MS. At this stage, neurological dysfunction progressively worsens without phases of remission occurring. Clinical manifestation of progressive MS following RRMS is termed secondary progressive MS (SPMS). If the disease onset is progressive from onset on, however, it follows the course of primary progressive MS (PPMS) (Figure 1).

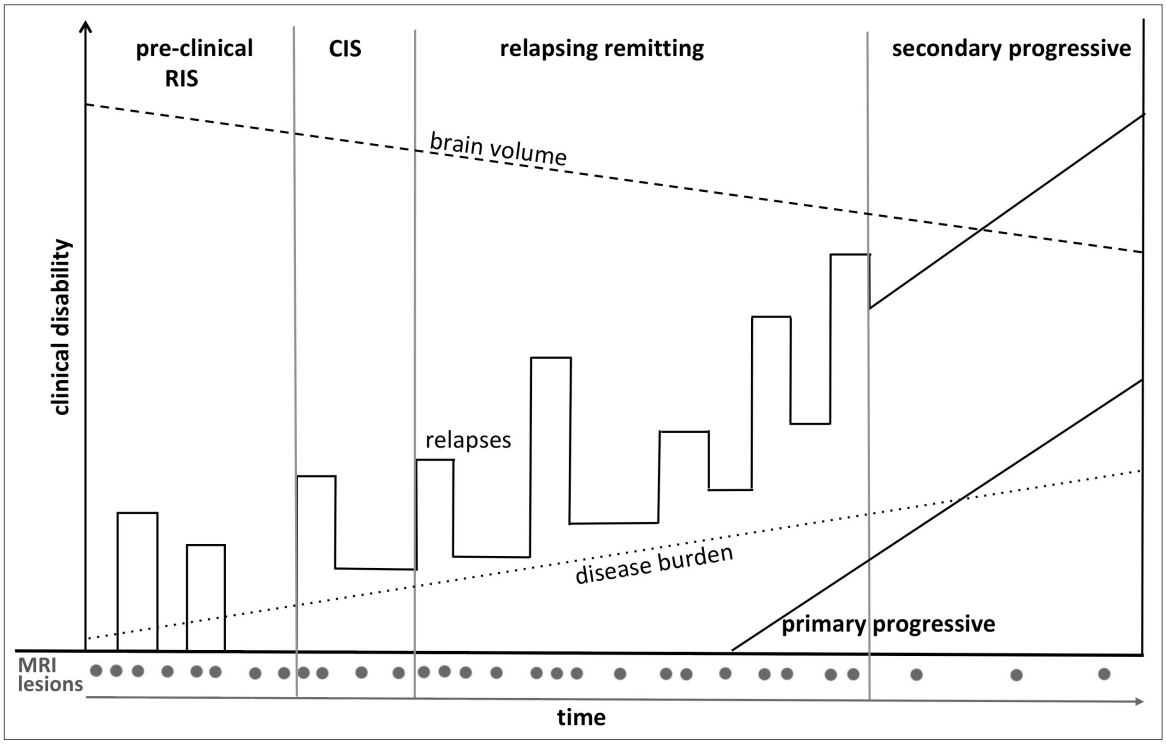

Figure 1: Stages and disease progression in Multiple Sclerosis. The course of MS shows an overall increase of disease burden accompanied by an increased clinical disability and decreasing brain volume over time. The pre-clinical stage includes the radiological isolated syndrome (RIS), which is often asymptomatic and incidentally discovered on MRI scans. RIS develops into the clinical stage, clinically isolated syndrome (CIS). CIS is followed by a relapsing remitting course of disease (RRMS) during which symptoms with periods of relapses and neurological dysfunction as well as pro-inflammation manifest and get replaced by periods of remission. The final clinical stage is secondary progressive MS (SPMS), which evolves from RRMS and lacks remission phases. If disease onset progresses from the beginning, it is termed primary progressive MS (PPMS). Brain volume and atrophy is measured by Magnetic Resonance Imaging (MRI) scans as a biomarker for MS providing information about newly occurring, active and persistent brain lesions and with that disease progression over time. (tailored based on [2])

To date, MRI scans of the CNS are the most common biomarker for MS disease activity [2]. Using MRI scans, active, newly occurring and persistent lesions can be detected,

visualized and observed over time allowing the measurement of disease burden and progression. CNS lesions and inflammation are a hallmark of MS and are accompanied by a blood brain barrier (BBB) leakage allowing the scarcely restricted infiltration of immune cells from the peripheral blood stream [1]. One of the first cell populations found in lesions inside the CNS include lymphocytes [3]. Therefore, MS is presumed to be of autoimmune etiology initiated by over- and auto-reactive T lymphocytes finally targeting myelin sheaths of neurons. This process of BBB leakage and CNS infiltration has been described especially in RRMS patients and leads to the diverse clinical symptoms.

The main symptoms patients present with include muscle weakness, fatigue, impairments in motor function and coordination, cognitive impairments and major depressive disorder (MDD). MDD is the most prevalent psychiatric comorbidity in MS patients with a lifetime prevalence of developing depression of up to 50% [4]. Studies have shown strong associations between an increased inflammatory profile in MS patients with increased fatigue and depression scores further contributing to the inflammatory processes observed in MS patients [5]–[7]. Importantly, symptoms can be very diverse and depend on the degree of disability and overall disease burden of a patient.

Causes for the onset of MS are multifaceted and include genetic and environmental factors, sex differences and hormone regulation (comprehensively reviewed in [8]).
While the kind of MS and disease severity do not show a genetic component, with first-degree relatives having a 2-5% higher risk for developing MS, disease susceptibility seems to relate to genetic effects. Numerous studies showed that most genes associated with MS seem to relate to either the innate or adaptive immune system. One of the most dominant risk factors is the HLA-DRB1*15:01 allele in the *class II major histocompatibility complex* (MHC) gene [9]. Examples for non-MHC associated MS-susceptibility loci are the *interleukin-7 receptor* α (IL7-Rα or CD127) and *interleukin-2 receptor* α (IL2-Rα or CD25) genes. Genome wide association studies (GWAS) detected small nuclear polymorphisms (SNP) in the *IL7R*α gene in MS patients, where the SNP most likely induces an increased abundance of soluble IL7-Rα protein [10]. The IL7-Rα is essential in T and B lymphocyte maturation, development and homeostasis and downstream activation of the janus kinase (JAK) and signal transducer activators of transcription (STAT) pathways- both involved in gene transcriptions following immune responses and in direct interaction with

the nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NFκB) pathway [11]. The IL2-Rα is upregulated on activated T cells and therefore essential in cellular proliferation, survival and T cell homeostasis [12],[13]. Taken together, genetic risk factors for MS hint toward strong associations with immune regulation, however, no single genes have been defined to be involved in causing MS.

Another contributor to MS development and progression are environmental influences. One of the most widely discussed environmental factor in MS disease is the protective effect of ultra violet (UV) radiation exposure. Especially early in life before the age of 20, elevated UV radiation exposure and with that increased vitamin D levels have been shown to reduce MS-risk later in life [14]. Vitamin D signaling greatly affects anti-inflammatory immune cell pathways via T and B lymphocyte suppression and regulatory T cell (T regs) induction, with that promoting protective immune tolerogenic effects [15]. Vitamin D levels have also been shown to have protective effects in other autoimmune diseases like type 1 diabetes (T1D), systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [16], indicating it being an essential influence in immunity.

The effects of sex differences and hormones have also been studied extensively in MS. The 2.5:1 ratio of females:males affected by MS is striking. This ratio has been explained with parent of origin effects where, for example disease associations like the risk allele HLA-DRB1*15:01 show a stronger association with females [17]. Additionally, X- and Y-chromosome-linked genes have been shown to contribute to gene-dosage effects and immune regulation of innate and adaptive immune responses [18]. Furthermore, hormone regulation greatly affects cells of the innate and adaptive immune system displaying distinct differences and adaptations in females and males (particularly estrogen and testosterone, respectively). This effect is most prominent, considering the strong protective effect of pregnancy from relapses [19].

Taken together, genetics, environmental influences, sex and hormones allow a deeper understanding of MS disease and help to better comprehend the underlying causes leading to the complex pathogenesis.

1.1.2 Current Treatment

Treatment for MS includes medications to treat occurring symptoms as well as basic immune-modulatory medication. Symptom management aims to increase the quality of life and reduce MS symptoms. Especially during relapses, patients oftentimes are treated with cortisone to manage peripheral and CNS inflammation.

Current immune-modulatory medications to treat RRMS are targeted at innate and adaptive immune cells. They suppress pro-inflammatory signaling and have become more advanced in the last decade with to date more than ten drugs approved by the American Food and Drug Administration (FDA). Baecher-Allan and colleagues most recently published an extensive review summarizing current drugs, their specific targets and the mechanisms of action [2]. In summary, the modes of action include the trapping of T lymphocytes inside the lymph nodes and with that prevention of T lymphocyte migration via the BBB into the CNS, the promotion of T regs and decrease of MS-disease inducing Th1/Th17 cells as well as the inhibition and depletion of B lymphocytes. Considering efficacy, side effects, relapse history and CNS lesions, the clinician and patient decide which drugs to use. The efficacy of the drug is measured by the reduction in relapses and a decrease of inflammation. Using MRI scans, disease progression and brain atrophy is determined [20].

Almost all of the FDA approved drugs target the relapsing inflammatory stage and are largely not effective in the progressive forms of MS. Furthermore, to date, medications are effective in limiting relapses, but do not fully prevent disease progression.
Broadening the understanding of the molecular mechanisms of immune activity in MS may allow a deeper understanding of causative mechanisms underlying the disease.

1.2 Principals of the Immune System

The human immune system is composed of an enormous multitude of mechanisms all prepared to target threats to the body and fight infections. It entails molecular structures, cells, tissues and organs whose main function is the elimination of pathogenic agents as well as aged or cancerous body cells.

The immune response requires the regulation and interaction with other body systems, like the central nervous system and (neuro-) endocrine mechanisms as well as energy demand and response signaling pathways. It is classified into innate and adaptive immunity, which constantly interact to initiate effective defense mechanisms involving humoral (non-cellular) and cellular structures. The cellular components of the blood arise from pluripotent hematopoetic stem cells (HSC) in the bone marrow that possess the ability to develop into common lymphoid progenitor cells (T and B lymphocytes and natural killer cells) as well as common myeloid progenitor cells (monocytes, dendritic cells, granulocytes). Peripheral blood mononuclear cells (PBMCs) are composed of about 25-60% $CD4^+$ T lymphocytes, 5-30% $CD8^+$ lymphocytes, 5-10% B lymphocytes, 10-30% NK cells, 5-10% monocytes and 1-2% dendritic cell [21]. The various cell types and their functions within innate and adaptive immunity are subsequently discussed in detail.

1.2.1 The Innate Immune System

Innate immune response mechanisms are coded in the germline and compromise the first line of defense against pathogens like bacteria, viruses, worms or fungi. Examples for innate immune cells are neutrophils, basophils, eosinophils (all granulocytes), natural killer (NK) cells and monocytes. They circulate within the peripheral blood stream and constantly monitor for invading pathogens. Humoral components include the complement system, cytokines, chemokines and pattern recognition receptors (PRRs). Cytokines are small proteins involved in cell-specific signaling and communication. A subtype of cytokines are chemokines, which possess the ability to recruit cells to the site of inflammation. Both, cytokines and chemokines can be secreted by cells and bind to their specific cytokine and chemokine cell surface receptors. PRRs can also be membrane bound or soluble, recognizing conserved pathogen associated molecular patters (PAMPs). Examples for highly conserved PRRs are toll like receptors (TLRs). To date, more than 13 TLRs have been identified [22], each able to recognize one or multiple ligands, e.g. microbial motifs and debris from necrotic cells.

The recognition of PAMPs induces an immediate immune response. The release of cytotoxic molecules by innate immune cells induces the killing of extracellular pathogens or apoptosis (programmed cell death) of infected cells. Chemokines and cytokines recruit

additional immune cells causing further pro-inflammatory responses and pathogen clearance.

Additionally, most innate immune cells function as antigen presenting cells (APCs). Following the recognition by their TLR, APCs can present processed antigen via their MHC class II for antigen recognition by T lymphocytes of the adaptive immune system. Furthermore, MHC-II presentation can also occur after phagocytosis, the engulfment of cells or particles, of pathogens or infected cells. The interaction of innate and adaptive immune cells and their humoral components is a central element of immune response mechanisms.

Phagocytosis and antigen processing as well as the release of cytotoxic molecules are two central elements in innate immunity. NK cells and monocytes are two cell subpopulations involved in these processes and are subsequently described in more detail.

1.2.1.1 Natural Killer cells

NK cells are granular lymphocytes of the innate immune system essential in anti-bacterial and anti-viral immune responses [23]. They predominantly detect cells that lack MHC class I expression and with that the identification of a cell as self. The main functions of NK cells include the secretion of immune activating cytokines and chemokines and the killing of infected or transformed cells via pathways including perforin/granzyme or death receptor-related pathways [24]–[26]. They express TLRs involved in the recognition of bacterial and viral PAMPs (e.g. TLR2, TLR5, TLR7/8, TLR9) [22] and also play a central role in tumor immunity [25] and human pregnancy [27].

The activation of NK cells by TLR ligands is accompanied by the production of interferon γ (INFγ) and granulocyte-monocyte colony stimulating factor (GM-CSF) and requires the presence of assessor cytokines like IL-2 and IL-12, IL-2 and IL-18 or IL-15 and IL-18 [28]. Consequently, NK cell activation is coupled to assessor processes safeguarding over-reactivity.

Based on their function, cytokine secretion profile and cell surface receptor expression, NK cells are subdivided into regulatory and cytotoxic subtypes. Regulatory NK cells are characterized as $CD56^{+}$ $CD16^{-/low}$ and are mainly found in lymph nodes, lacking cytotoxic

potential. Cytotoxic NK cells are defined as $CD56^{+}$ $CD16^{+/high}$, are more differentiated and have the potential to mediate cytotoxicity [29].

INFγ is the signature cytokine of $CD56^{bright}$ NK cells and known to shape Th1-mediated adaptive immunity [26] as well as to induce macrophages to kill intracellular pathogens and activate APCs to up-regulate MHC class I molecules [30]. Additionally, INFγ secretion by NK cells has anti-proliferative effects on cells that have been transformed due to viral infection or malignancies [31].

Taken together, NK cells play an important role in innate immune response and cellular activation as well as in the induction of adaptive immune mechanisms.

1.2.1.2 Monocytes

Monocytes are central APCs of the innate immune system. They get released into the periphery where three monocyte subpopulations have been characterized based on their cell surface receptor expression: classical monocytes ($CD14^{++}$ $CD16^{-}$), intermediate monocytes ($CD14^{++}$ $CD16^{+}$) and non-classical monocytes ($CD14^{+}$ $CD16^{++}$) [32]. After circulating in the blood, they reach tissue where they differentiate and further mature into tissue resident dendritic cells (DC) or macrophages.

The main functions of monocytes include pro-inflammatory cytokine production following the recognition and phagocytosis of pathogens and the antigen presentation via MHC class I and II molecules. The signature cytokines released include TNFα, IL-1 and IL-6 [33].

Monocytes express numerous TLRs (e.g. TLR2, TLR9) for antigen recognition [34],[35] and phagocytosis. The latter takes place by direct binding to pathogens via PAMP recognition or by detection of antibody- or complement-coated pathogens (opsonization). Unlike NK cells, monocytes do not require the presence of assessor cytokines for their activation.

Once they are activated, they release pro-inflammatory cytokines and with that induce the transmigration of other innate and adaptive immune cells to the site of inflammation. Depending on the intruding pathogen, monocytes have been shown to be able to induce helper T cell mediated immune responses and with that the activation of the adaptive immune system [33]. Moreover, monocytes have been shown to act as suppressor cells inducing Treg development and inhibiting T lymphocyte proliferation [36].

Taken together, monocytes display an essential role in rapid innate immune response mechanisms and are key components in adaptive immune cell activation, proliferation and suppression.

1.2.2 The Adaptive Immune System

The adaptive immune system compromises defense mechanisms that are acquired, develop from the time of birth and evolve over the course of a lifetime. As described for innate immune responses, adaptive immunity also entails cellular and humoral components. However, contrary to innate immunity, adaptive immunity is highly antigen-specific. Main cell types constituting adaptive immunity include T and B lymphocytes. T lymphocyte subpopulations include helper and cytotoxic T lymphocytes with specific cell surface receptor expression and antigen-specific functions. B lymphocytes produce antigen-specific antibodies, which are part of the humoral structures in adaptive immunity. Furthermore, both, T and B lymphocytes, possess the ability to form memory cells after antigen clearance, a crucial process that allows an immediate immune response during a secondary infection with the same antigen.
The ability of specialized cell subpopulations to respond to specific antigens and generate immune memory are key characteristics of adaptive immunity and are subsequently described in more detail.

1.2.2.1 T lymphocytes – development, differentiation and activation

T lymphocytes originate from stem cells in the bone marrow. Once released, they travel to the thymus (thymocytes) and mature into functional $CD4^+$ and $CD8^+$ T lymphocytes (subsequently termed $CD4^+$ and $CD8^+$ T cells). In the process of thymic maturation, a broad repertoire of antigen-specific T cell receptors (TCR) is established.
Most TCRs entail an α- and a β-chain (>85% of T cells) and only the remaining fraction is made up of γ:δ-TCRs. The TCR protein structure comprises a constant unmodified region and a variable region. The variable region is the site of antigen-recognition and specific for each T cell. During T cell development, the α and β genes of the variable region of the TCR undergo somatic recombination of the variable-joining (V-J, α-chain) and *variable-*

diversity-joining (V-D-J, β-chain) gene segments. This process allows the tremendous diversity of the TCR antigen-recognition repertoire, which is estimated with approximately 10^{11} specificities [37].

The TCR is part of a group of a membrane-bound CD3-protein complex, whose interaction is required during antigen recognition. The CD3-complex is made up of a γ and δ chain as well as two ε and two ζ chains. Additionally, T cell co-receptors are required for antigen-recognition and T cell activation, including CD40L (CD154), CD28 and CD4 (on $CD4^+$ T cell subpopulations) or CD8 (on $CD8^+$ T cell subpopulations) [38].

During thymic maturation, thymocytes are selected based on their ability to recognize self and foreign antigen via their TCR. Briefly, during the first major stage of thymic T cell differentiation, double-negative $CD4^-$ $CD8^-$ T cells undergo TCR rearrangement. This allows them to progress to the second major stage of differentiation where they are termed double-positive T cells expressing both CD4 and CD8 co-receptors. At this point, APCs present a multitude of antigens and self-peptides via MHC classes I and II to the premature T cells. Now, positive and negative selection occurs during which highly self-reactive T cells (binding to self-antigens on the MHCs) and T cells that do not bind MHC are eliminated. T cells that are weakly reacting to self-peptides receive survival signals allowing them to mature into the third stage of differentiation. During this stage, either the CD4 or CD8 receptor is down-regulated yielding single-positive (SP) naïve $CD4^+$ $CD8^-$ T cells and SP naïve $CD4^-$ $CD8^+$ T cells. They emigrate from the thymus into the blood stream as well as secondary lymphoid organs (like the spleen, Peyer's patches in the gut, mucosal tissue, tonsils or adenoids). Their main function is immune surveillance. Once they encounter their specific antigen, they mature into functional helper or effector T cells.

T cell activation requires the binding of the antigen-specific TCR-complex to the MHC-antigen complex on the cell surface of APCs. The MHC-complex is composed of the co-stimulatory receptors CD80 or CD86 (binding to CD28 on the TCR) as well as CD40 (binding to CD40 L on the TCR). $CD4^+$ T cells recognize antigens from pathogens like extracellular bacteria, worms or toxins that have been processed by APCs and presented on their MHC class II. In contrast, $CD8^+$ T cells recognize antigens from intracellular bacteria, viruses and endogenous molecules like tumor proteins that have been processed by APCs and presented on their MHC class I.

Taken together, the activation of T cells requires the binding of the TCR-complex to the MHC-antigen complex including all co-receptors. T cell activation induces a cytokine and chemokine environment (also involving other immune cells), which results in the differentiation into specific $CD4^+$ helper T cell subsets and $CD8^+$ effector T cells as well as the recruitment of other immune cells to the site of inflammation.

Following an antigen encounter, memory T cells are formed. They possess the ability to undergo clonal expansion, proliferation as well as activation of other immune cells inducing a fast immune response to a previously intruding antigen. Three memory T cell subsets are classified based on their function and expression of surface receptors: central memory T cells (T_{CM}), effector memory T cells (T_{EM}) and terminally differentiated effector memory cells re- expressing CD45RA (T_{EMRA}) (summarized in Table 1). Upon activation and differentiation to memory T cells, $CCR7^+$ naïve T cells (T_N) down-regulate the CD45RA isoform and express the CD45RO isoform. T_{CM} are $CCR7^+$, have high proliferative capacities and lower effector functions. T_{EM} are $CCR7^-$, resident in peripheral tissues and possess the ability to quickly adapt a $CD4^+$ effector or $CD8^+$ cytotoxic T cell phenotype. The third memory subtype are T_{EMRA}, which are $CCR7^-$, show low proliferative and functional capacities and characteristics of senescent cells [39].

Taken together, $CD4^+$ and $CD8^+$ memory T cell subpopulations monitor and patrol the body for intruding pathogens in tissues and the periphery. They are highly effective and time-efficient during a secondary immune response to a previously targeted pathogen in the primary immune response. This is the essence of adaptive immunity allowing pathogen-specific immune responses.

1.2.2.1.1 $CD4^+$ T lymphocytes

Upon activation, $CD4^+$ T_N differentiate into effector T cells including helper T cell (Th) types Th1, Th2, Th17 and Th1/17. Helper T cells mediate cellular and humoral immune responses specific to the intruding pathogen. They are characterized based on their chemokine receptor and cytokine profile as well as individual transcription factors (Table 1 provides a summary). An additional $CD4^+$ T cell subpopulation are regulatory T cells

(Treg), which complement immune activation and are essential for immune homeostasis and self-tolerance.

Th1 cells produce IFNγ and are activated and induced by interleukin (IL)-12 as well as the transcription factors T-bet, STAT1 and STAT4 [40]. Th1 cells are characterized based on the chemokine receptors CXC-chemokine receptor (CXCR) 3 and chemokine receptor (CCR) 5 [41]. A Th1-mediated immune response leads to the targeted reaction to intracellular bacteria and the activation of infected macrophages within the inflammatory micro-environment.

Th2 cell development is promoted by IL-4 as well as the transcription factors STAT-6 and GATA-3 [42]. Characteristic transcription factors are CCR4 and Chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTh2) [43]. They secrete IL-4, IL-5, IL-9 and IL-13 and stimulate histamine- and heparin-secreting mast cells contributing to the elimination of extracellular pathogens like bacteria or worms. Furthermore, Th2 cell stimulation leads to the activation of B lymphocytes inducing antigen-specific antibody production.

Th17 cells induce a strong pro-inflammatory immune response to extracellular pathogens like bacteria and fungi. They are characterized based on CCR4 and CCR6 chemokine expression [41]. Cytokines promoting the induction of Th17 cells include IL-6, IL-17, IL-23 and transforming growth factor-β (TGF-β), which also induce the expression of the Th17-specific transcription factors retinoic acid receptor-related orphan nuclear receptor (ROR) γt, RORα and STAT-3 and vice versa [42]. Following pathogen intrusion, Th17 cells induce neutrophil activation, which promotes a fast line of defense via the release of anti-microbials, the elimination of pathogens by phagocytosis and the induction of chemotaxis. Th17 immune response mechanism can be inhibited by Th2 cells, thereby preventing an overreaction of the immune system and with that a damage of self-tissue.
Th1/17 cells have been described as a transitional state between Th17 and Th1 cells producing IFNγ and IL-17 [41]. The conversion of Th1 to Th17 cells has been shown to impact both, MS disease progression as well as regulatory functions [44].

T regs are classified as Forkhead box $P3^+$ (FoxP3) $CD25^+$ $CD4^+$ T cells. They are primarily generated during thymic selection (referred to as tT regs or natural T regs) and possess a relatively high affinity to self-antigens on the border to being negatively selected [45].

Additionally, peripheral T regs (pT regs) can be generated extrathymically and induced T regs (iT regs) can be generated in cell culture stimulated by transforming growth factor-β (TGF-β). The transcription factor FoxP3 is the master regulator of T regs and along with CTLA-4 essential for T reg functionality. Importantly, T regs express no or only low levels of IL-7 (CD127), while $CD4^+$ $CD25^-$ $CD127^+$ conventional T cells (all $CD4^+$ T cells but T regs) express IL-7Rα on their surface [46]. IL-10, IL-35 and TGF-β are the signature cytokines secreted by T regs and induce T reg activation and proliferation. T regs are crucial for the suppression of excessive immune responses as well as the maintenance of immunological unresponsiveness to self-antigens.

Table 1 provides a comprehensive overview of the $CD4^+$ T helper cell and memory T cell subtypes including the chemokine receptor expression profiles characteristic for each phenotype. It summarizes the previously described subsets and their descriptive transcription factors, cytokine profiles, as well as natural functions.

Table 1: CD4$^+$ helper T cell and memory cell subtypes, transcription factors, cytokine profiles, phenotype and natural function. Modified based on [39],[43],[47]**.**

T cell subset	TF	inducing cytokines	secreted cytokines	pheno-type	natural function
Th1	T-bet	IL-12	IFNγ, GM-CSF	CXCR3$^+$ CCR5$^+$	cell-mediated immunity, intracellular pathogens
Th2	GATA3	IL-4	IL-4, IL-5, IL-10, IL-13	CCR4$^+$ CRTh2$^+$	humoral immunity, extracellular parasites
Th17	RORγt	IL-1β, IL-6, IL-23, TGF-β	IL-17, GM-CSF	CCR6$^+$ CCR4$^+$	mucosal immunity, extracellular fungi and bacteria
Tregs	FoxP3	-	TGF-β	CD25$^+$, CD127$^-$	immune homeostasis, (self-)tolerance
T_N			IL-2	CD45RA$^+$ CCR7$^+$	progenitor cells, immune surveillance
T_{CM}			IL-2, IL-21	CD45RO$^+$ CCR7$^+$	immune memory, B cell help, secondary expansion
T_{EM}			IL-4, IL-5, IL-17, TNF-α, IFNγ	CD45RO$^+$ CCR7$^-$	immune memory, B cell help, protection in tissues
T_{EMRA}				CD45RA$^+$ CCR7$^-$	immune memory, terminally differentiated cells

IL: interleukin, TF: transcription factor, Th: helper T cell, IFN-γ: interferon γ, GM-CSF: granulocyte-macrophage colony-stimulating factor, mTOR: mechanistic target of rapamycin, ERR: estrogen receptor, HIF-1α: hypoxia-inducible factor-1α, FoxP3: forkhead P3, TGF: transforming growth factor, AMPK: AMP-activated protein kinase, T_N: naïve T cells, CM: central memory, EM: effector memory, T_{EMRA}: terminally differentiated effector memory cells re- expressing CD45RA, TNF-α: tumor necrosis factor-α, CCR: Chemokine Receptor, CXC: CXC-Chemokine Receptor, CRTh2: Chemoattractant receptor-homologous molecule expressed on Th2 cells, RORγt: retinoic acid receptor-related orphan nuclear receptor γt.

1.2.2.1.2 CD8$^+$ T lymphocytes

CD8$^+$ T cells are a T lymphocyte subpopulation that, in contrast to CD4$^+$ T cells, recognize MHC class I molecules on APCs via their CD8 TCR complex and are very potent pro-inflammatory immune activators (described in 1.2.2.1). They differentiate and proliferate into cytotoxic CD8$^+$ T cells inducing a cascade of pro-inflammatory signaling to fight off

the intruding pathogen or neoplastic cells. Signature cytokines of cytotoxic T cells include IL-2, TNF-α and INF-γ. Additionally, due to their function in clearing intracellular viruses, bacteria and cancer cells, $CD8^+$ T cells secrete perforin, granzyme and granulolysin inducing a strong immune attack. To prevent an immune overreaction, cytotoxic T cells require at least three signals to become activated: TCR specific antigen recognition, co-receptor binding (e.g. CD28) and IL-2 signaling (which may include Th cells that provide IL-2) [48].

To date, three main $CD8^+$ cytotoxic T cell subpopulations have been described. They are differentiated based on their chemokine expression and are unique to their locations in the body. CCR4, CCR6 and CXCR3 are used for their characterization. The specific immune functions of the subpopulations have been studied to a greater extend, but still remain somewhat elusive. $CD8^+$ $CCR4^+$ $CCR6^-$ $CXCR3^-$ T cells are predominantly found in the epithelium and lung [49] and are involved in Th2-mediated immunity [50]. $CD8^+$ $CCR6^+$ $CCR4^-$ $CXCR3^-$ T cells are mainly located in the gut and have been shown to be essential in Th17-mediated immune responses, while $CD8^+$ $CXCR3^+$ $CCR4^-$ $CCR6^-$ T cells display key immune functions in Th1 immunity [50].

$CD8^+$ cytotoxic T cells act on pathogen infected cells or neoplastic cells by releasing granzyme, granulolysin and perforin. These factors are released from granules within the cytoplasm of cytotoxic T cells and are exerted onto the target cells. They induce apoptosis, form holes in the target cell membranes, inhibit virus replication and act as anti-microbials by creating a pro-inflammatory micro-environment.

1.2.2.2 B lymphocytes - development, differentiation and activation

Just like T cells, B lymphocytes (subsequently termed B cells) are lymphocytes derived from pluripotent hematopoetic stem cells in the bone marrow and are part of the adaptive immune system. B cells and the immunoglobulins (Ig, also known as antibodies) they produce make up an essential part of humoral immunity protecting against an enormous variety of pathogens. The B cell antibody repertoire entails antibodies that can recognize more than $5x10^{13}$ specificities [51]. B cells can produce five different types of immunoglobulins: IgM, Ig,A, IgG, IgD and IgE. All of them differ in size, specificity and functionality [51].

The protein structure (variable and constant regions) as well as the genetic mechanisms underlying the production of antibodies resemble that of the TCR on T cells. Briefly, during cellular development in the bone marrow, B cells undergo the following stages: stem cell, early pro-B cell, late pro-B cell, large pro-B cell, small pre-B cell and immature B cell [52]. When the precursor forms of the B cells undergo these stages, rearrangements of the variable (V), joining (J) and diversity (D) regions of the heavy chain and VJ rearrangement of the light chain occurs. Heavy-chain rearrangement occurs after the early pro-B cell stage and its success is tested selecting for functional heavy chains. If the cell passes that first functionality checkpoint, it undergoes light-chain rearrangement followed by a second checkpoint selecting for functional light chains. If that succeeds, the cell moves on to the immature B cell stage.

Following these briefly summarized developmental stages, the immature B cell possesses an antigen-specific immunoglobulin (Ig), which is embedded into the cell membrane and part of the B cell receptor (BCR) complex. Immunoreceptor tyrosine-based activation motifs (ITAMS) are linked to Ig on the BCR and induce activation signaling upon antigen-specific immunoglobulin binding [53].

Immature B cells that recognize self-antigens in the bone marrow are eliminated, rescued or become tolerant. The process of central tolerance includes BCR editing, clonal elimination, depletion, inactivation or anergy. Immature B cells that do not recognize self-antigens in the bone marrow migrate into the lymphatic system as immature transitional B cells where they undergo the process of peripheral tolerance after encountering their BCR specific antigen. They either become anergic, undergo clonal deletion or move on to mature. BCR-specific antigen recognition as well as IgM and IgD expression characterizes mature naïve B cells. Mature naïve B cells are further characterized as $CD20^{+}$ $CD3^{-}$ lymphocytes [54] and have been described to secrete the immunosuppressive cytokine IL-10 [55]. CD20 is expressed from the pre-B cell stage until the mature naïve as well as memory B cell stage, however, plasma cells lack CD20. Mature naïve B cells circulate between lymphoid vessels and the blood stream where they encounter APCs as well as helper T cells and patrol for intruding pathogenic antigens.

Interestingly, mature B cells undergo a process termed somatic hypermutation. In addition to immunoglobulin gene rearrangement in the BM, this highlights a second step in B cell antigen recognition diversification. During somatic hypermutation, high-

frequency point mutations occur at the hypervariable sites of the variable regions on the light and heavy chains. These sites are the points of contact with antigens. Therefore, the affinity of the antibody to an antigen can get increased or decreased allowing antibodies with medium or low antigen affinities to become highly-specific for pathogenic antigens [51]. Somatic hypermutation is specific to B cells and has not been described in T cells.

Compared to T cells, B cells do not depend on MHC presentation for activation. B cells can recognize and bind antigen directly via their BCR along with co-receptors and have the ability to bind non-protein antigens like foreign polysaccharides and DNA independent of T cell engagement [56]. Nevertheless, B cells can bind antigens by their antigen-specific BCR. The BCR-antigen complex gets engulfed and the foreign antigen is processed and subsequently presented on the cell surface via MHC class II for recognition by e.g. helper or memory T cells. B cells are essential APCs for helper T cells and initiate helper T cell activation, expansion as well as differentiation. Similarly, B cells may depend on helper T cell-mediated initiation of activation, proliferation and differentiation following antigen presentation by T cells [57]. Once the B cell recognizes its specific antigen, the cell undergoes clonal expansion and develops into an antibody-producing plasma cell fighting off the pathogen and activating additional immune mechanisms and cells of the innate and adaptive immune system.

In addition to B cells undergoing clonal expansion, antigen-specific memory B cells are formed, which able to effectively and efficiently fight re-occurring antigens [58]. These long-lived memory B cells are $CD20^+$ and able to persist for years [59]. They have been described to be $CD27^+$ and produce pro-inflammatory cytokines like IL-12, lympho toxin α (LTα) and TNF-α [55].

In summary, B cells are very potent antigen-specific lymphocytes with a tremendous antigen recognition repertoire that is vital in complementing T cell immune responses.
The described developmental stages and numerous functions of B cells require the fine interaction and modulation of cellular energy pathways. Here, the ability to switch between low and high energetic states is fundamental and allows successful immune cell functionality. The following text discusses the central role of metabolism in immune cell function.

1.3 Immunometabolism

The word *metabolism* is derived from the Greek word *μεταβολισμός* (*metavolismós)*, which means *change*. Immune cells have numerous specifications, functionalities and responsibilities and with that need to display very distinct metabolic profiles to meet their individual tasks and fulfill their target functions. Therefore, the ability to adapt and change their metabolic profile is crucial for healthy immune response mechanisms and host survival.

One of the first researchers analyzing cellular metabolic function was Otto Warburg. In 1956, he described that even under aerobic conditions cancer cells heavily depend on glycolysis instead of oxidative phosphorylation (OXPHOS) to meet their increased energy demand- an observation that has subsequently been termed the Warburg Effect [60],[61]. From there on, the analysis of cell-specific energy pathways has spread to all kinds of research areas including immunology.

Subsequently, the main cellular energy generating pathways are described and linked to immune cell function.

1.3.1 Main Cellular Energy Pathways

Cells are regulated by extrinsic and intrinsic mechanisms and therefore modulate their specific activities and metabolic pathways based on their requirements for survival, growth or development. The ability to use and switch between different pathways to generate energy from nutrients like sugars, proteins and fats are key characteristics of cellular energy metabolism. Typically, the energy is stored in the chemical bonds of these nutrients and the catabolism (break down) of these bonds releases energy, which is used to generate energy rich molecules like adenosine triphosphate (ATP) that the cell can subsequently use.

Importantly, cellular chemical reactions have exceptionally high reactive potential and are therefore always profoundly controlled for. Energy signaling pathways in eukaryotic cells, including human immune cells, occur either in the cellular cytosol or within mitochondria. The cytosol is defined as the liquid inside the cell and is surrounded by the cell membrane. Glycolysis, the pentose phosphate pathway (PPP) and amino acid (AA)

metabolism including glutaminolysis take place in the cytosol. It separates numerous compartments within the cell, with distinct functions and responsibilities. One of these specialized compartments within eukaryotic cells are mitochondria. Mitochondria are thread like structures that are highly adaptable to cellular energy needs. They are the site of the tri-citric acid (TCA) cycle as well as the electron transport chain (ETC) and OXPHOS. Mitochondria are made up of an inner and an outer mitochondrial membrane with the inter-membrane space in between. The inner membrane forms engulfings (cristae) into the inside of the mitochondria, the mitochondrial matrix. Cristae are the site of ETC complexes and energy production in the form of ATP.

To meet the cell's energy demand, mitochondria possess the ability to adapt their morphology. They can fuse together, a process termed mitochondrial fusion and observed to be required in fatty acid oxidation (FAO) and memory T cell development [62],[63]. During fusion, mitochondrial cristae move further apart and thereby decrease ETC efficiency and energy production in the form of ATP. Additionally, the division of mitochondria, termed mitochondrial fission, has been observed in effector T cells and increases mitochondrial energy production. Fission induces mitochondrial cristae, and with that ETC complexes, to move closer together and thereby allows for a greater efficiency of energy production, specifically, ATP generation [62],[63].

Fusion and fission are also required for the regulation of mitophagy. Mitophagy has first been described by Lemaster and colleagues as the process of degradation of damaged mitochondria [64]. In cellular division, fission processes allow the equal distribution of mitochondria between cells, induce the segregation of damaged mitochondria and initiation of mitophagy [65],[66]. A most recent comprehensive review by Williams and Ding summarizes the complex field of mitophagy and the underlying molecular mechanisms and pathophysiological roles [67]. Briefly, the authors describe nuclear and mitochondrial genes involved in mitophagy processes and the relevance of mitophagy processes in innate and adaptive immunity. E.g., in T cells, mitophagy is essential for cellular differentiation and mitophagy deficiency has been shown to increase mitochondria abundance, toxic reactive oxygen species and with that lead to overall impaired peripheral T cell survival [68]. The relevance of mitophagy with respect to auto-immune diseases requires future investigations.

Mitochondria possess their own mitochondrial DNA (mt-DNA) as well as ribosomes for protein synthesis. Mt-DNA is believed to have its origin in the endosymbiotic theory and encodes about 37 genes that are coding for ETC molecules and amino acids [69],[70]. In sexual reproduction, mitochondria are inherited via the egg cell from the mother.

Mitochondria-associated membranes (MAMs) directly connected mitochondria to the endoplasmic reticulum (ER) and the nucleus allowing immediate and efficient communication between the cell structures. Among other molecules, lipids and calcium ions (Ca^{2+}) can be shuttled between the ER and mitochondria, which is essential in energy metabolic homeostasis [71].

An overview of the major metabolic pathways is given in Figure 2. Glycolysis, the PPP, FAO, glutaminolysis, the TCA cycle, the ETC and OXPHOS are shown as the main energy producing pathways within the cell.

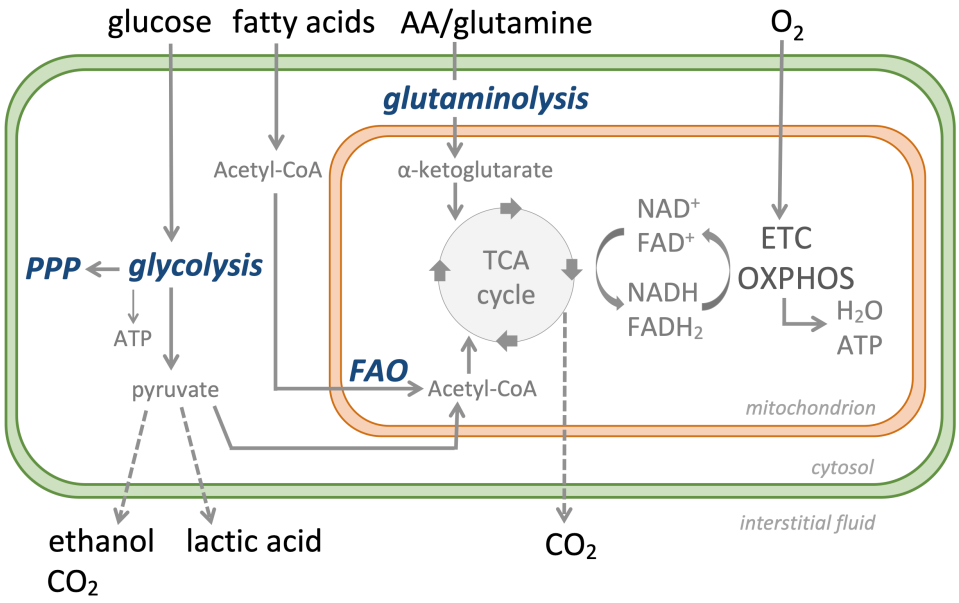

Figure 2: Overview of main cellular energy pathways. In energy production pathways, nutrients are taken up from the interstitial fluid outside the cell into the cytosol. Subsequently, they get catabolized via numerous chemical reactions yielding energy-rich ATP, NADH and $FADH_2$ molecules. Glycolysis, PPP, glutaminolysis (all taking place in the cytosol), FAO, ETC and OXPHOS (all occurring in the mitochondria) are the main pathways during which glucose, fatty acids and glutamine are broken down. OXPHOS requires the presence of molecular oxygen (O_2). Metabolic waste products like carbon dioxide (CO_2), ethanol and lactic acid are secreted from the cell. AA: Amino Acids, ATP: Adenosine Triphosphate, ETC: Electron Transport Chain, $FADH_2$: Flavin Adenine Dinucleotide H_2, FAO: Fatty Acid Oxidation, NADH: Nicotinamide Adenine Dinucleotide H, OXPHOS: Oxidative Phosphorylation, PPP: Pentose Phosphate Pathway, TCA: Tri-citric Acid Cycle. (designed based on [62],[63],[72]–[76])

Each signaling cascade is described in the subsequent text passages beginning with FAO.

1.3.1.1 Fatty Acid β-oxidation and the Mitochondrial Membrane Protein CPT1a

Fatty acid β-oxidation (short β-oxidation or FAO) describes the catabolism of fatty acids to numerous products that the cell can use for energy production. The starting point of this reaction is the beta carbon of the fatty acid molecule, hence the name β-oxidation.

In order for FAO to commence, fatty acids need to be activated and subsequently transferred from the cytosol via the mitochondrial membrane into the mitochondrion. The activation of fatty acids involves an enzyme-mediated reaction in the cytosol during which acetyl-coenzyme A (acetyl-CoA) is bound to the fatty acid. Short-chain fatty acids (between 2 and 6 carbon atoms in size) can diffuse into the mitochondria. However, medium- and long-chain fatty acids (larger than 6 carbon atoms) cannot diffuse into mitochondria and require a specific transporter [72],[74].

The central molecule facilitating this transfer is CPT1a (Carnitine palmitoyltransferase I isoform a), which is located in the mitochondrial membrane. CPT1a is a member of the carnitine/choline acetyltransferase family and located on chromosome eleven [49]. The mitochondrial trans-membrane protein forms homohexamer and homotrimer complexes with Acyl-CoA Synthetase Long Chain Family Member 1 (ACSL1) and Voltage Dependent Anion Channel (VDAC) 1 and has also been found in complexes with VDAC2 and VDAC3 [49].

CPT1a activates fatty acid by conjugating medium- and long-chain fatty acids to carnitine. This allows the conjugated fatty acid acyl-CoA to be shuttled into the mitochondrion where it is transferred back to fatty acid acyl-CoA by CPT2 (Carnitine palmitoyltransferase 2) [76]. Now, FAO can begin yielding acetyl-CoA, NADH (Nicotinamide Adenine Dinucleotide H) and $FADH_2$ (Flavin Adenine Dinucleotide H_2). The lipid synthesis intermediate malonyl-CoA inhibits CPT1a once fatty acid synthesis (FAS) occurs in a nutrient abundant environment. Therefore, the transfer of fatty acids via CPT1a is the rate-limiting step and CPT1a the key regulatory protein in FAO.

Compared to glycolysis, FAO is very efficient and allows the generation of large amounts of ATP from complex fatty acid molecules. For example, the complete FAO of one palmitate molecule can produce more than 100 ATP molecules, while one glucose molecule can only yield up to 36 ATP molecules [79].

The generated NADH and $FADH_2$ molecules from FAO are electron donors for the ETC and finally OXPHOS for the generation of ATP, which is subsequently described in more detail.

1.3.1.2 Electron Transport Chain and Oxidative Phosphorylation

During oxidative phosphorylation (OXPHOS), electrons are transported down an electrochemical proton gradient of the electron transport chain (ETC). The ETC is made up of complex I to complex V and is integrated into the inner mitochondrial membrane [80] [81]. It is a series of redox (reduction and oxidation) reactions performed by numerous enzymes during which electrons are transferred from electron donors to acceptors, allowing the transfer of hydrogen protons (H^+) from the mitochondrial matrix to the inter-membrane space. This generates a proton gradient, which is used for ATP synthesis. Importantly, the process of ADP (adenosine diphosphate)-phosphorylation to form energy-rich ATP requires the presence of molecular oxygen (O_2)- hence it is termed OXPHOS. Therefore, the generation of energy via the ETC is a process of aerobic respiration and an oxygen-dependent metabolic pathway.

The five complexes of the ETC (Figure 3) each have distinct properties and complement each other. In complex I, also termed NADH dehydrogenase, two electrons are removed from the electron donor NADH. In addition to NAD^+, the redox reaction yields hydrogen protons (H^+), which are pumped into the mitochondrial inter-membrane space. The reaction also yields electrons, which are transported to the next complex in the ETC, complex II. The function of complex I can be inhibited by e.g. rotenone (Table 2) preventing the electron transfer to complex II and causing an accumulation of electrons in the mitochondrial matrix as well as the inhibition of hydrogen proton transfer into the inter-membrane space.

Complex II (succinate dehydrogenase) is made up of four subunits and the succeeding complex in the ETC. In addition to electrons derived from complex I, complex II receives electrons from electron donors like succinate via its cofactor $FADH_2$. This complex does not contribute to the proton transfer into the inter-membrane space, however transfers its electrons to the next complex in the ETC:

In complex III (termed bc1 complex (ubiquinol:cytochrome c oxidoreductase), a series of redox reactions yields electrons that are transferred to complex IV via cytochrome c.

Additionally, hydrogen protons are pumped into the inter-membrane space further contributing to the build up of the electrochemical proton gradient. Complex III can be inhibited by antimycin A (Table 2).

The succeeding complex in the ETC is complex IV. The transmembrane protein complex is also known as cytochrome c oxidase. It receives electrons from cytochrome c of complex III and transfers them to molecular oxygen, which yields a H_2O molecule. During this process, hydrogen protons are transferred into the inter-membrane space.

The uneven difference in hydrogen proton concentration between the mitochondrial matrix and inter-membrane space is used by ATP-synthase, complex V, of the ETC. It constitutes two regions with numerous subunits and is the final step of the ETC during which ATP-generation occurs from ADP and phosphate. The hydrogen protons are passed via the ATP-synthase into the matrix allowing ATP synthesis to occur. The process of ADP-phosphorylation can be disrupted by the chemical agent FCCP (Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone) (Table 2), which is an uncoupling agent transporting hydrogen protons into the mitochondrial matrix, thereby disrupting the proton gradient and with that preventing the process of ATP synthesis.

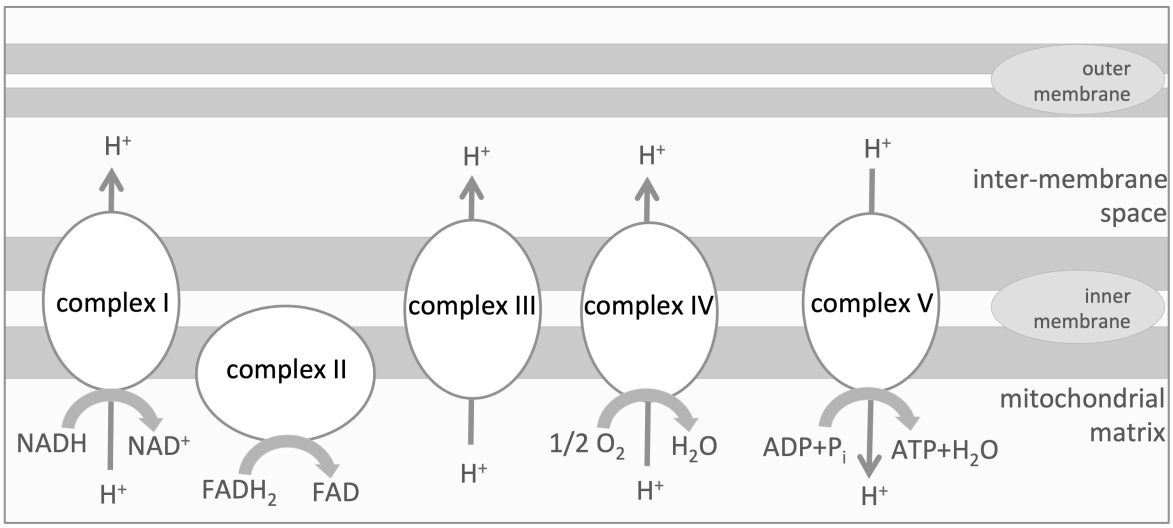

Figure 3: Display of the mitochondrial electron transport chain. The electron transport chain (ETC) is located at the inner membrane of mitochondria. Electrons are donated to complex I of the ETC and transported in a series of redox reactions to complex II, III, IV and V. This allows the transfer of hydrogen protons (H^+) from the mitochondrial matrix to the inter-membrane space generating a proton gradient between the mitochondrial matrix and the inter-membrane space. This proton gradient is used for ATP synthesis by complex V. ADP: Adenosine diphosphate, ATP: Adenosine triphosphate, P_i: inorganic phosphate, FAD: Flavin Adenine Dinucleotide, $FADH_2$: Flavin Adenine Dinucleotide H_2, H^+: Hydrogen proton, H_2O; water molecule, NAD: Nicotinamide Adenine Dinucleotide H, NAD: Nicotinamide Adenine Dinucleotide, redox: reduction and oxidation reaction, O_2: oxygen. (designed based on [80])

Table 2: Inhibitors of mitochondrial electron transport chain complexes. Table adapted from [82]–[85].

inhibitors	mechanism of toxicity
oligomycin	inhibits ATP-synthase (complex V) and induces a hyperpolarization of the inter-membrane space
FCCP	uncouples mitochondrial inner membrane, inhibits ATP synthesis by allowing free flow of H^+ between the inter-membrane space and matrix
rotenone	inhibits complex I-induced NADH oxidation to NAD
antimycin A	complex III inhibition

In summary, the ETC and OXPHOS are a series of efficient and highly specialized reactions within the mitochondrion that require the presence of molecular oxygen and yield tremendous amounts of energy-rich ATP molecules that the cell can use for any metabolic reactions. Predominantly, this energy is received from the oxidation of fatty acids that yield NADH and $FADH_2$ molecules. Furthermore, in addition to FAO, energy can be generated from glucose molecules in a process termed glycolysis.

1.3.1.3 Glycolysis and the Glucose Transporter GLUT1

Glycolysis takes place in the cytosol and is a key metabolic pathway during which glucose ($C_6H_{12}O_6$) is catabolized in consecutive reactions into pyruvate along with numerous byproducts. The glycolytic pathway begins with the uptake of extracellular glucose from the interstitial fluid, the immediate cellular microenvironment surrounding the cell. Glucose molecules require a transporter in order to cross the cell membrane. One of the main trans-membrane glucose transporter proteins with a high affinity of glucose is Glucose transporter 1 (GLUT1), which is encoded by the gene *Scl2a1*. It is part of a family of glucose transporters including GLUT1 to GLUT14, each of which expressed in specific body tissues and cells [75].

The intracellular processing of glucose in the glycolytic pathway entails a series of enzymatic reactions yielding pyruvate molecules. Under anaerobic conditions, pyruvate can be processed into lactate and ethanol. Under aerobic conditions, pyruvate is processed to acetyl-CoA and entered into the TCA cycle, which takes place in the mitochondrial matrix. Anaerobic glycolysis can occur even though oxygen is abundantly

present in the cell, e.g. during inflammatory processes or in cancer cells (Warburg effect). However, anaerobic glycolysis is less efficient in the generation of energy-rich ATP. The catabolism of one glucose molecule yields two ATPs during anaerobic glycolysis and 36 ATPs if processed via the TCA cycle and ETC respiration [76]. Lactate and CO_2 are waste products that are secreted by the cell and are associated with an increased acidic microenvironment [86].

Nevertheless, there are central benefits of glycolysis, including the generation of the co-factor NADH, which is essential in cellular anabolism. Furthermore, glycolysis provides intermediates for the biosynthesis of fatty acids, amino acids as well as ribose for nucleotides. Consequently, cellular dependency of glycolysis has been observed in growing, activated and proliferating cells, which require large amounts of biomaterial.

1.3.1.4 Tri-citric Acid Cycle

An additional major cellular metabolic pathway is the tri-citric acid (TCA) cycle, also known as the Krebs cycle named after Hans Krebs, who discovered it in 1957 [87]. The TCA cycle takes place in the matrix of mitochondria and receives products from multiple nutrients. Acetyl-CoA from glucose-derived pyruvate or fatty acids and α-ketoglutarate from glutamate metabolism enter the cycle at different stages. In a series of reactions, the TCA cycle yields NADH and $FADH_2$ that transfer their electrons to the ETC for the generation of ATP during OXPHOS (reviewed in Figure 2).

The TCA cycle, along with the ETC and OXPHOS, is a highly efficient energy pathway that is predominantly performed by cells that are quiescent or non-proliferating and whose main requirement is longevity [76]. However, if specific growth signals are present, they can promote the production of amino acid and lipids from TCA cycle intermediates. This modification of TCA cycle reactions requires nutrient abundance and diversion to the cycle. The process of amino acid and lipid production is also observed in the glycolytic pathway [76].

1.3.1.5 Pentose Phosphate Pathway

The Pentose Phosphate Pathway (PPP) takes place in the cytosol and uses intermediates from the glycolytic pathway (Figure 2) for the production of nucleotide and amino acid precursors (e.g. for DNA and RNA synthesis) as well as NADPH (phosphorylated NADH) [73], which is an essential electron donor and important antioxidant. The PPP supports cellular survival, growth and proliferation. The pathway does not require or produce ATP and is oxygen-independent.

1.3.1.6 Amino Acid and Glutamine Metabolism

Amino acids are essential building blocks for protein synthesis associated with the PPP and are used as substrates in different metabolic reactions, e.g. FAS. The amino acid Glutamine can be taken up by the cell, converted to glutamate and finally α-ketoglutarate, which is entered into the TCA cycle for energy production (Figure 2). Additionally, it can serve as a source of citrate for *de novo* fatty acid synthesis in anabolic metabolism and cellular growth [73].

Taken together, the cellular energy metabolism pathways comprehensively described here, are used by cells to meet their individual metabolic requirements for cellular survival, development and functionality. Specific regulatory mechanisms regarding energy metabolism in immune cells are examined in the subsequent text.

1.3.2 Regulation of Immune Cells by Adapting Energy Metabolism

As discussed in *1.2*, innate and adaptive immune cells are in constant contact and exchange with their immediate microenvironment. They are part of complex interactions e.g. via their cell-specific receptors or secreted cytokines. All of these mechanisms serve the purpose of inter-cell communication, activation and support once pathogenic agents or abnormal cells are detected. Non-activated or resting immune cells predominantly rely on OXPHOS and ETC for energy production since they do not depend on broad anabolic processes. An activated immune cell requires an increased energy production with upregulated metabolic pathways to meet the extensive pro-inflammatory signaling and

anabolic processes. Therefore, upon activation, cells switch to increased glycolysis rates for energy production, increased TCA cycle activity, glutaminolysis as well anabolic processes including the PPP. These mechanisms of increased energy pathways in activated cells are observed in adaptive immune cells like T cell and B cells as well as in innate immune cells like macrophages or NK cells [63],[76]. Depending on the kind of energy metabolism the cell relies on, it is able to perform specific functions. Without the switch to an elevated energy metabolism, an immune cell could not implement pro-inflammatory signaling.

Figure 4 shows a brief overview of resting and active state T cell characteristics. T cells that are in a more resting state include naïve T cells, memory T cells and resting regulatory T cells. These cell populations are in a metabolically latent state and switch to increased immunometabolism once they become activated [88]. This increase in metabolic function is crucial for $CD4^+$ helper T cell, $CD8^+$ effector T cell and activated regulatory T cell function [73],[74] and allows memory T cells to undergo clonal expansion. Along with elevated immunometabolism, particularly glycolytic activity, intracellular and extracellular pH levels increase due to elevated lactate and CO_2 secretion from the cell. Furthermore, reactive oxygen species (ROS) increase indicating considerable cellular activity and stress. Importantly, in healthy individuals, immune cells are able to modify resting and active states depending on the immediate requirements. After antigen clearance, $CD4^+$ helper T cells, $CD8^+$ effector T cells and regulatory T cells are able to return to their resting state. This leads to a homeostasis in energy metabolism that allows the appropriate function of immune response mechanisms and down-regulation of pro-inflammatory signaling following an immune attack.

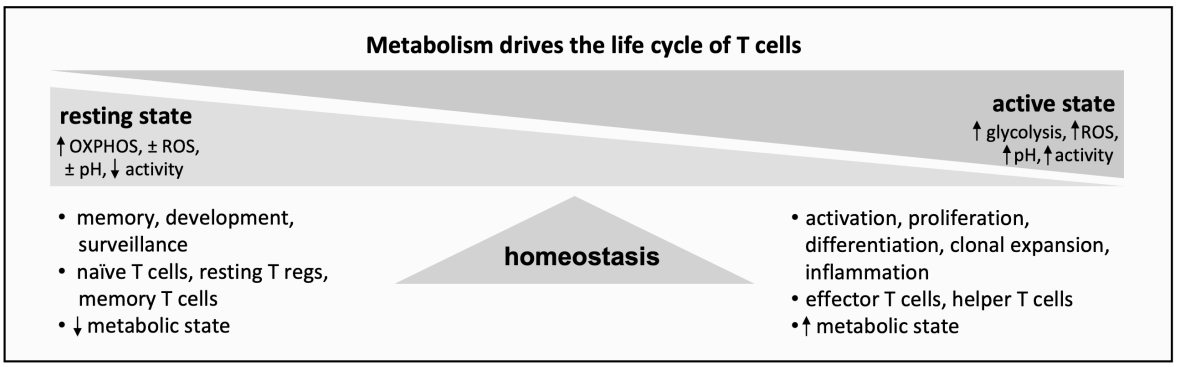

Figure 4: Metabolism drives the life cycle of T cells. Overview of metabolic states in activated and resting T cells. Resting T cells are more prone to using OXPHOS and FAS for meeting their energy demand. ROS levels are not elevated and pH is not decreased. Naïve T cells, resting T regs and

memory T cells are exemplarily for resting T cell metabolism. Their overall activity is lower than in activated cells. Cells in an activated metabolic state are relying more on glycolysis and FAO. Their ROS as well as pH levels are increased - activated, proliferating, differentiating cells and cells undergoing clonal expansion switch to this active state. These cells include effector and helper T cells. Their overall metabolic sate is increased. OXPHOS: oxidative phosphorylation, FAS: fatty acid synthesis, FAO: fatty acid oxidation, ROS: reactive oxygen species, T reg: regulatory T cells.

The maintenance of naïve, effector, regulatory and memory T cells requires cell-specific signaling. Table 3 provides a detailed overview of the characteristic metabolic programs as well as the key metabolic regulators of $CD4^+$ T cell subtypes essential for the preservation of the cell type. Key metabolic signaling molecules in pro-inflammatory T cell activation include mTOR (mammalian target of rapamycin), the hormone receptor ERRα (estrogen receptor α) and the transcription factor HIF-1α (hypoxia-inducible factor-1α) (helper T cell subpopulations). AMPK (AMP-activated protein kinase) acts in immune-suppressive and anti-inflammatory signaling pathways. These metabolic regulators play central roles in immunometabolic cell signaling cascades and are essential in T cell homeostasis.

Table 3: $CD4^+$ T cell subtypes and energy metabolic features. *Modified based on* [39],[43],[47],[74]**.**

T cell type	key metabolic regulator	metabolic program
T_N	not known yet	OXPHOS/glycolysis
Th1	mTORC1/ERRα	glycolysis/FAS/AA met.
Th2	mTORC2/ERRα	glycolysis/FAS/AA met.
Th17	mTORC1/ERRα/HIF-1α	glycolysis/FAS/AA met.
T regs	AMPK	OXPHOS/FAO
T_{CM}, T_{EM}, T_{EMRA}	AMPK and others	OXPHOS/FAO

OXPHOS: oxidative phosphorylation, FAS: fatty acid synthesis, FAO: fatty acid oxidation, AA met.: Amino Acid metabolism, Th: helper T cell, mTOR: mechanistic target of rapamycin, ERR: estrogen receptor, HIF-1α: hypoxia-inducible factor-1α, FoxP3: forkhead P3, AMPK: AMP-activated protein kinase, T_N: naïve T cell, CM: central memory, EM: effector memory, TEMAR: terminally differentiated effector memory cells re-expressing CD45RA.

Just like $CD4^+$ T cell subsets, $CD8^+$ T cells rely on adaptations in energy metabolic pathways and show comparable metabolic profiles. Naïve $CD8^+$ T cells predominantly use OXPHOS and glycolysis to meet their energy demand [89]. The high active state of $CD8^+$ effector T cells demands elevated energetic requirements. Therefore, effector T cells

heavily depend on glycolysis, FAS and amino acid (AA) metabolism to meet their energy demand. In contrast, memory $CD8^+$ T cells require less energy and depend on FAO and OXPHOS to meet their energy requirements [90],[91]. Key metabolic regulators in $CD8^+$ T cell subsets are believed to be similar to $CD4^+$ T cell subsets, but have yet to be described in detail in literature.

Adaptations in energy metabolism of B cells and innate immune cells have been reviewed extensively in [92]–[94]. Comparable to the mechanisms observed in T cell subpopulations, B cells and innate immune cells show increased glycolysis and FAS in pro-inflammatory cell signaling and FAO as well as OXPHOS in latent and non-proliferative cell populations.

Taken together, immune cells are in constant exchange with their metabolic regulatory systems in order to respond to the cellular requirements and defend the host against intruding pathogenic agents.

To date, broad research has shown the interplay between metabolism and immunity in healthy individuals and research is evolving in analyzing pathogenic alterations in disease states. Here, autoimmune diseases like MS are of central interest, because dysfunctional immune activation processes may show direct links to impaired metabolic signaling.

Importantly, the interplay of physiological and psychological stress response systems as well as exhaustion of T cells due to prolonged stimulation and activation are key factors contributing to the pathology and symptoms of MS and have to be taken into consideration when discussing the essential interplay of metabolism in MS disease.

1.3.3 HPA-axis Stress Response Signaling

One of the main stress response systems in the human body is the hypothalamus-pituitary-adrenal (HPA) axis, which involves a cascade of mechanisms of hormone secretion by tissues of the brain and kidney.

The HPA axis is activated upon an encounter of an individual with a physiological or psychological stressor. It commences with the secretion of corticotropin-releasing hormone (CRH) by neuroendocrine neurons of the hypothalamus. Secondly, CRH stimulates adrenocorticotropic hormone (ACTH) secretion by the anterior lobe of the

pituitary gland. Lastly, ACTH acts on the adrenal cortex of the adrenal gland inducing glucocorticoid (GC) synthesis. The adrenal gland predominantly secrets the GC cortisol, which, in a negative feedback loop, has the potential to act on the hypothalamus and pituitary gland preventing CRH and ACTH secretion. For clinical analysis, cortisol levels are mainly measured in saliva samples from individuals.

GCs are one of the most potent hormones modulating pro- and anti-inflammatory processes in the body and bind the glucocorticoid and mineralocorticoid receptors. The glucocorticoid receptor (GR) is found in the cytosol of various body cells including neurons and immune cells. In immune cells, GCs induce anti-inflammatory signaling pathways and with that immunosuppression either by direct binding to proteins or by regulating gene expression of specific targets [95]. One of the major mediators of immunosuppression via GCs is the anti-inflammatory protein GC-induced leucine zipper (GILZ), which binds and inhibits one of the main pro-inflammatory signaling molecules, NFκB [96],[97]. The comprehensive mechanisms by which GCs induce anti-inflammatory actions in cell signaling pathways are reviewed extensively by Libert and Dejager [98].

In healthy individuals, the process of cortisol release and GC signaling allows the suppression of immune responses in favor of the activation of the sympathetic nervous system and the physiological and psychological stress response. Once the stressor is absent, cortisol levels decrease. During the day, cortisol levels peak in the morning before awakening and decrease over the course of the day with the lowest levels at night initiating sleep.

In autoimmune patients, alterations in HPA axis activity in combination with increased disease levels have been shown alluding to a link between neuronal stress hormone signaling and immune cell function. As reviewed by Gold *et al.*, hyperactivity of the HPA axis as well as decreased T cell sensitivity to GC signaling contributes to MS disease onset, development and progression [99]. To date, GCs have been shown to impact helper T cell activation, differentiation and proliferation, which are key processes in immune response mechanisms and metabolic functionality [100]. Furthermore, the prolonged stimulation and activation of T cells is associated with cellular exhaustion mechanisms, which can be driven forward by systemically altered stress response systems.

The relevance of T cell exhaustion in the context of immunometabolism is discussed in the subsequent text.

1.3.4 T cell Exhaustion

T cell exhaustion describes the process of prolonged cellular stimulation over weeks and months. It coincides with the expression of specific inhibitory cell surface receptors, for example cytotoxic T lymphocyte associated protein 4 (CTLA-4), T cell immunoglobulin and mucin domain containing protein 3 (TIM-3), B and T lymphocyte attenuator (BTLA), lymphocyte activation gene 3 (LAG-3) or programmed cell death-1 (PD-1) [101],[102]. T cell exhaustion can be induced during or after chronic infections and cancer involving persistent and ongoing antigen exposure and overall inflammation. The state of T cell exhaustion prevents the induction of an optimal immune response to infection or tumor. However, this state has been shown to be reversible and immune re-activation possible, if the pathways involved in T cell exhaustion are modulated (e.g. via PD-1) [103],[104]. In their 2015 review, Wherry and Kurachi discuss the evolutionary and biological significance of the state of T cell exhaustion [105]. Exemplarily, the authors point out that exhausted T cells are not inert and have been shown to posses specific functions like the containment of chronic infections, the potential of driving epitope mutation in chronic infections, or the prevention of tissue damage. Overall, from an evolutionary standpoint, a host-pathogen balance is established in persisting infections allowing immune control and preventing uncontrolled pathogen replication. Importantly, inhibitory cell surface receptors are crucial regulators or auto-reactivity and are essential in preventing autoimmunity [106].

Immunometabolic signaling and stress response systems are key characteristics contributing to exhaustion as well as overall altered immune cell function and need to be considered in autoimmune diseases like MS.

1.4 Multiple Sclerosis and Immunometabolism

In the past decades, extensive research demonstrated that immune cells of MS patients are self-reactive and cause dramatic damage in the body over long periods of time with phases of remission and relapses in RRMS patients. As described previously, the precise causes of MS and factors leading to relapses in RRMS are not fully understood yet (1.1.1), however, research results strongly suggest dysregulated pro-inflammatory immune cell

processes. Activated immune cells require altered metabolic pathways and signaling cascades and therefore have to be considered in MS disease development and progression.

T cells have successfully become one of the main targets for therapies and mechanisms of action of prospective drugs continue to be studied extensively. Immunometabolic profiles of immune cells from MS patients, especially of T cell subtypes, need to be considered and the impact of energy metabolism recognized as a potent disease influencing factor and potential target for therapies in the future.

To date, immunometabolic function has mostly been researched in the murine model of MS, the experimental autoimmune encephalomyelitis (EAE). In mice, EAE can be induced by immunization with myelin-specific antigen and adjuvants [107],[108]. EAE presents with complex immunopathological and neuropathological conditions that have been shown to be comparable to the key pathological symptoms observed in MS, namely systemic inflammation and CNS neuronal demyelination [108]. In the murine model, several factors benefiting a positive course of disease and decline of symptoms as well as complete reduction of EAE disease have been observed. For example, fasting (no food intake for up to 16 hours) and ketogenic diet (strong reduction of carbohydrates in diet) have proven to alleviate EAE symptoms, reduce pro-inflammatory cytokines as well as antigen presenting and Th1 and Th17 T cells, while inducing an increase of T regs [109]. These positive effects of adaptations in diet have also been studied in murine models of other autoimmune diseases like Systemic Lupus Erythematosus (SLE) and have proven to benefit the disease course [110]. A comprehensive review describing the link between metabolism and immune function including cytokine and hormone effects by specific innate and adaptive immune cells was recently published by Alwarawrah et. al in 2018 [111]. The authors describe in detail that impaired metabolic signaling interplays with immune function, is linked to autoimmunity and possesses potentials for therapy in autoimmune diseases. Furthermore, the positive effects of autophagy in inflammatory processes and auto-immunity have been described [112]. Autophagy is defined as an evolutionary conserved process of cellular self-renewal by lysosomal degradation of macromolecules thereby eliminating waste products and maintaining the metabolic homeostasis [113]–[115]. Proteins associated with autophagy processes orchestrate responses to nutrient deprivation as well as pathogens. First studies describe the positive

contributions of a fasting diet to cellular autophagy processes and anti-inflammation with that indicating the potential for reestablishing impaired metabolic balance in immune cells [116]–[119].

To date, there are few studies on the mechanisms of immunometabolism in MS disease in humans. Impairments in mitochondrial respiration and glycolytic activity has been observed in PBMCs from MS patients [120]. In 2015, De Riccardis and colleagues showed impairments in $CD4^+$ T cells from RRMS patients with decreased mitochondrial respiration and increased glycolytic activity [121]. These studies provide first insights into human immune cell metabolism in RRMS patients and allude to overall impairments in energy metabolism.

Along with energy metabolic pathways, the HPA axis stress response system has shown strong interactions with immune cell activation and pro-inflammatory signaling. Cortisol binds to the GR of immune cells and induces decreased pro-inflammatory signaling cascades. Importantly, in immune cells, the frequent induction of the GR can lead to the down-regulation of the receptor by the cell. Thereby, the cell possesses the ability to escape the induction of anti-inflammatory signaling via the HPA axis and cortisol hormone. In MS patients, alterations in HPA axis activity have been shown, including the down-regulation of the GR and associated proteins [122]. Alterations in stress response systems and pro-inflammatory signaling in immune cells impact cellular energy metabolism and should therefore be considered in autoimmunity.

These first studies in murine models and MS patients strongly allude to overall impairments in immunometabolic cell signaling and energy transduction pathways. They provide firm data for future analyses of metabolic dysfunctions in MS disease.

Particularly, mitochondrial function in T cells of MS patients in the context on pro-inflammatory cell signaling needs to be studied and its potential for therapeutic targets. Here, inflammatory signaling, cellular exhaustion pathways and stress response mechanisms including CNS hormone signaling strongly impact energy pathways and need to be studied collectively when analyzing MS disease development and progression.

1.5 Aims

The aim of this dissertation was the investigation of human immune cell subpopulations from RRMS patients linking energy metabolism pathways, mitochondrial function, inflammatory signaling and stress response mechanisms.

Energy metabolism profiles of $CD4^+$ T cells, $CD8^+$ T cells and non- $CD4^+/CD8^+$ T cells were compared between RRMS patients and HC participants. Three main aims were defined, as shown graphically in Figure 5.

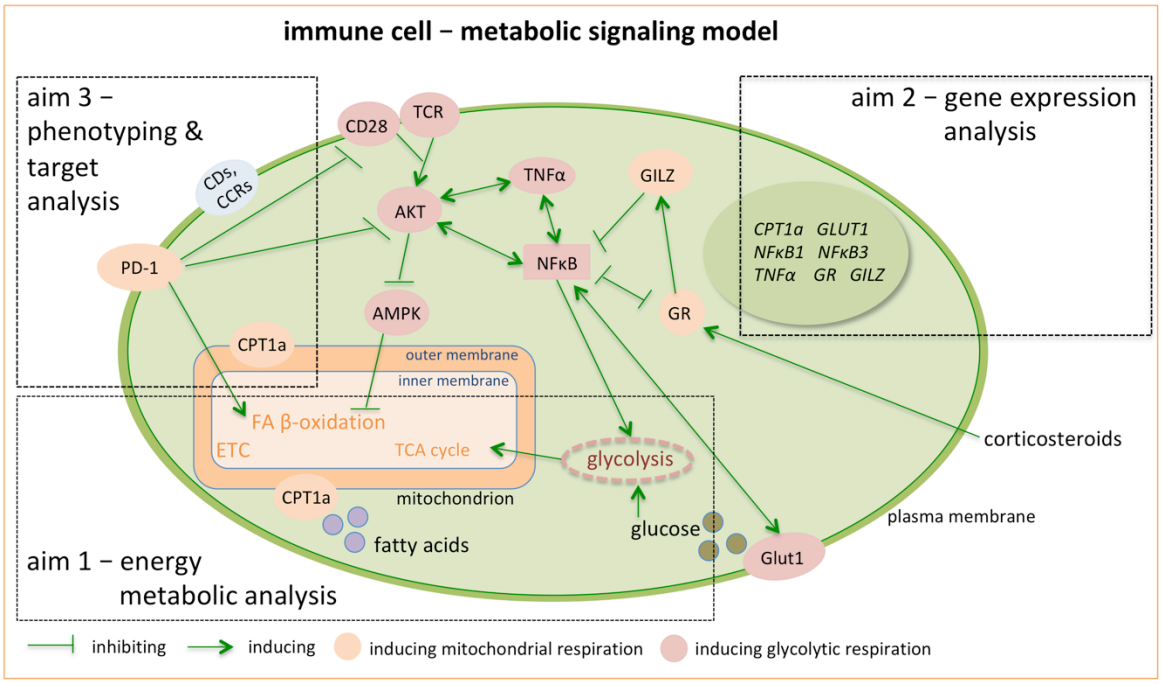

Figure 5: Model of Immunometabolic Signaling. The model includes three aims that were analyzed as part of this dissertation. Aim 1: energy metabolic analyses using the Seahorse 96^eXF Analyzer and mitochondrial stress test kit. Aim 2: gene expression mRNA analysis of *CPT1a, GLUT1, NFκB1, NF-κB3, TNFα, GR, GILZ.* Aim 3: flow cytometry analyses of chemokine receptors as well as PD-1 and CPT1a. Molecules in red show glycolysis-inducing signaling, molecules in orange demonstrate an induction in mitochondrial respiration. AKT: protein kinase B, AMPK: AMP-activated protein kinase, CPT1a: Carnitine palmitoyltransferase I isoform a, CCR: chemokine receptor, CD: cluster of differentiation, ETC: electron transport chain, FA: fatty acid, GLUT1: glucose transporter 1, NF-κB: nuclear factor 'kappa-light-chain-enhancer' of activated B-cells, PD-1: programmed cell death-1, TCA: Tri-citric acid, TCR: T cell receptor, TNF-α: tumor necrosis factor-α.

First, mitochondrial respiration and glycolytic function in these three subpopulations was assessed using the Seahorse XF^e96 Analyzer providing information about mitochondrial activity. Secondly, qRT-PCR gene expression analyses provided information about key regulators in cellular inflammatory, metabolic and stress response signaling. Lastly, the

third aim, flow cytometry analyses provided data on the PBMC phenotype make-up (chemokine receptor analyses), extracellular PD-1 T cell exhaustion surface marker analyses as well as intracellular mitochondrial membrane protein CPT1a analyses.
These three aims link mitochondrial and glycolytic activity to gene and protein expression of key energy, inflammatory and cortisol pathway genes in human immune cells. They provide information about the immunometabolic networks and hormone regulators on a protein and gene expression level as well as providing information about the interaction with cellular respiration.

To gain biomaterial from RRMS patients and matched HC participants, the clinical study *Depression and Immune Function* (DENIM) was initiated and conducted as part of this dissertation. PBMCs were prepared for analyses and case report form assessments provided information about RRMS disease parameters as well as additional physiological and psychological disease indications. Furthermore, saliva samples from RRMS patients and HC participants were analyzed for cortisol levels providing information about HPA axis activity in both cohorts and linking immunometabolism to CNS hormone signaling.

2 Materials and Methods

2.1 Clinical Study Depression and Immune Function (DENIM)

2.1.1 Background and Aim of the Clinical Study DENIM

As described in 1.1, Multiple Sclerosis (MS) is a neurodegenerative disease strongly associated with auto-reactive immune cell activation. The main symptoms of MS include motor deficits, cognitive impairments, decreased ability to focus and concentrate, depression as well as an overall inflammatory phenotype.

The aim of the clinical study *Depression and Immune Function (DENIM)* was to gain first insights into the underlying biological mechanisms of immune cell activation and neuropsychiatric symptoms in patients diagnosed with RRMS with and without comorbid major depressive disorder (MDD). The main interest was the analysis of immunometabolic mechanisms related to physiological as well as psychological symptoms.

2.1.2 DENIM Study Organization and Set-up

2.1.2.1 Study Organization

The study was approved by the ethics committee of the Charité University Medical Center Berlin on May 21st 2015 (application number: EA1/096/15). Data protection of patient and healthy control (HC) participant information is guaranteed based on the Charité Data Protection Statement. The study is listed on www.clinicaltrials.gov with the ID: NCT02740296.

All MS patients were assessed at the Neuro Cure Clinical Research Center (NCRC) (head: Prof. Dr. med. Friedemann Paul) at the Charité Campus Mitte, Berlin. HC participants were assessed at the NCRC as well as at the Department of Psychiatry and Psychotherapy, work group Neuropsychiatry (head: Prof. Dr. Stefan Gold), at the Charité Campus Benjamin Franklin, Berlin.

2.1.2.2 Study Visit Set-up

DENIM is a cross sectional clinical study with a one-time study visit. MS patients and HC participants came into the clinic for the assessment, which included a blood draw, analysis of vital parameters, cognitive tests, demographic, psychological and physiological health questionnaires and a structured clinical interview assessing the psychological health. Furthermore, all patients and participants took saliva samples in the two days following the study visit. The visit was scheduled between 8 am and 10 am. Patients and participants came in for a fasting blood draw after not having eaten for 12 hours. The study was conducted in German and all questionnaires were in German. Patients and participants were all native German speakers or had language levels of a native speaker. An overview of all study visit assessments is given in Table 5 and complete case report form, telephone screening sheets and salivary sample collection sheets in Appendix.

2.1.2.3 Inclusion and Exclusion Criteria

The study analyzed RRMS patients and carefully matched HC participants. Men and women between the ages of 18 and 55 were included in the study. The DENIM study was set-up to analyze four patient cohorts: 1) MS patients with a confirmed diagnosis of RRMS and no current or past history of MDD (cohort RRMS), 2) MS patients with a confirmed diagnosis of RRMS and a current diagnosis of MDD (cohort RRMS+MDD), 3) patients suffering from MDD and no commodities of the immune system (cohort MDD) and 4) healthy control participants (cohort HC). For this dissertation, the cohorts RRMS and HC were recruited and analyzed and information given here are related to these two cohorts.

A full list of inclusion and exclusion criteria for MS patients and HC participants is provided in Table 4.

Table 4*: Inclusion and exclusion criteria of the clinical study Depression and Immune System for RRMS patients and healthy control participants.*

Inclusion criteria	Exclusion criteria
• confirmed diagnosis of RRMS; no immune modulatory therapy or stable immune modulatory therapy for at least 6 months	• Infection within the past 2 months (e.g. the flu, a cold, a stomach virus infection)
• Between 18 and 55 years of age	• Pregnancy
• BMI ≤ 30	• Epilepsy, Schizophrenia or other neurological or psychiatric diseases
• No steroid treatment for the past 3 months	• Rheumatism, Lupus or any other immune diseases
• No vaccination in the past 3 months	• Diabetes
• Fasting blood draw after not having eaten for 12 hours	• HIV or Hepatitis infection
	• Myocardial infarct or cardiovascular disease
	• Stroke
	• Head injury with lasting impairments
	• Treatment with any of these drugs: anti-psychotics, antidepressants, insulin, anti-epileptic medicines
	• Medicines, alcohol or drug abuse within the past year
	• Participant in an interventional study

HC: Healthy Control, RRMS: Relapsing Remitting Multiple Sclerosis, BMI: Body Mass Index, HIV: Human Immunodeficiency Virus.

2.1.2.4 Telephone Screening for Study Eligibility

An extensive telephone screening was conducted with every MS patient and HC participant in order to verify inclusion and exclusion criteria for the study (Table 4). The MS patients' screenings were performed by the same interviewer (Aline Tänzer, AT) and HC participants screenings by AT and trained staff. Taken together, approximately 450 MS patients and 300 HC participants were contacted.

2.1.2.5 Case Report Forms – Questionnaires, Assessments, Tests

Case Report Forms (CRF) were conceptualized based on the study aims as well as inclusion and exclusion criteria (Table 4). The assessments performed with the MS

patients and HC participants are listed in Table 5. Only the questionnaires specific for the analyzed cohorts in this thesis were investigated. HC participants did not perform the MS specific neurological tests Multiple Sclerosis Functional Composite (MSFC), Expanded Disability Status Scale (EDSS) and Hamburger Lebensqualitätsfragebogen (HALEMS). The CRFs were conceptualized in cooperation with the NCRC. Trained personnel performed all cognitive tests and clinical assessments (including AT).

Table 5: Clinical assessments of RRMS patients and HC participants in a study site visit in the clinical study Depression and Immune Function.

Cognitive Tests:
Multiple Sclerosis Functional Composite (MSFC [214])[a]
Expanded Disability Status Scale (EDSS [135])[a]
Oral Symbol Digit Modality Test (oral SDMT [134])
Demographical, Psychological and Physiological Questionnaires:
Demography questionnaire
Beck's Anxiety Index (BAI [133, 216])
Beck's Depression Index II (BDI-II [132, 217])
Fatigue Scale for Motor and Cognitive Function (FSMC [220])
Fatigue Severity Scale based on Krupp (FSS [221])
Hamburger Lebensqualitätsfragebogen (HALEMS [222])[a]
Childhood Trauma Questionnaire (CTQ [223])
Clinical Interview Assessment
Mini International Neuropsychiatric Interview German version 5.0.0 DSM-IV (M.I.N.I.) [131] additional questions from the Diagnostic and Statistical Manual of Mental Disorders Version 5 (DSM-V)
Montgomery-Åsberg Depression Rating Scale (MADRS [130])

[a] MSFC, EDSS and HALEMS were not assessed with or by HC participants. RRMS: Relapsing Remitting Multiple Sclerosis, HC: Healthy Control.

2.1.2.6 Biological Material Collected

For routine blood analyses, whole blood samples were analyzed by a routine blood diagnostic laboratory (Labor Berlin, Berlin). A summary of items analyzed is given in supplementary Table 19. For research analyses, whole blood was prepared by trained

scientists following strict standard operating procedures (see 2.3.1.1 Cryo-preservation of Peripheral Blood Mononuclear Cells). Saliva samples for cortisol analyses were completed by the MS patients and HC participants independently at home after given the appropriate instructions (see 2.3.5 ELISA Analyses of Salivary Cortisol Hormone and Appendix for CRF salivary collection sheet).

2.1.3 DENIM Study Recruitment Period and Outcome

The DENIM study was launched with the first patient site visit on November 24th 2015.
The study ended with the last patient out on June 1st 2017.

2.1.3.1 Recruitment Period

During the recruitment time, a total of 52 study visits for RRMS patients and 50 study visits of HC participants were performed. Of these, 14 RRMS patients were 'drop-out' patients, i.e. patients, who had to be excluded after the study visit, because the data showed current substance abuse, a previous MDD diagnosis, dysthymia or a psychosis – patient information that had not been revealed during the telephone screening.

2.1.3.2 Sample Analysis

Generally, about 80 mL of fresh whole blood was drawn from each MS patient and HC participant and between $8x10^7$ and $12x10^7$ Peripheral Blood Mononuclear Cells (PBMCs) could be isolated. Aliquots of $1x10^7$ cells/cryo tube were frozen and stored in liquid nitrogen. Saliva samples of MS patients and HC participants were prepared and stored at -20°C until analysis.
All CRF data were computerized and statistics were performed using SPSS (IBM). Furthermore, statistics and graphical figures were completed using GraphPad Prism (GraphPadSoftware Inc.).

2.2 Materials

All companies listed are located in Germany, except if noted otherwise.

2.2.1 Laboratory Equipment

A list of all laboratory equipment used for the dissertation is given in Table 6.

Table 6: Laboratory equipment.

Autoclave 24	Melag, Berlin
Centrifuge Megafuge 2.0 R	ThermoFisher, Life Technologies, Berlin
CO2-Incubator Hera cell 240	ThermoFisher, Life Technologies, Berlin
Flow Cytometer FACS Canto II™	Becton Dickinson, Heidelberg
GeneAmp® PCR Systems 9700	Applied Biosystems, ThermoFisher, Life Technologies, Berlin
Invers-Microscope Axiovert 10	Zeiss, Jena
NanoDrop™2000c Spectralphotometer	ThermoFisher, Life Technologies, Berlin
Non-CO_2 Incubator Biometra APT Line	Binder, Tuttlingen
pH-Meter 766 Calimatic	Knick, Berlin
Pipettes	Eppendorf, Hamburg
Quadro MACS Seperator	MACS, Miltenyi Biotech, Bergisch Gladbach
Sterile Laboratory Bench Hera Safe	ThermoFisher, Life Technologies, Berlin
Step One Plus Real Time PCR Systems	Applied Biosystems, ThermoFisher, Life Technologies, Berlin

2.2.2 Glass and Plastic Equipment

A list of all glass and plastic ware used for the dissertation is provided in Table 7.

Table 7: Glass and plastic equipment.

Cell Culture Flask 275 mL	Sarstedt, Nürmbrecht
Cell Culture Plate 6-well	Greiner bio-one, Frickenhausen
Cell Cryo 1°C freezing container, Mr. Frosty™	Nalgene®, Roskilde, Denkmark
Cell Strainer 35 µm FACS tubes	ThermoFisher, Life Technologies, Berlin
Cryo Tubes	ThermoFisher, Life Technologies, Berlin
Eppendorf Reaction TZZSubes (1.5 & 2 mL)	Sarstedt, Nürmbrecht
Flow Cytometry tubes	Sarstedt, Nümbrecht
Glass Pipettes	Sarstedt, Nürmbrecht
MACS LS Colums	MACS, Miltenyi Biotech, Bergisch Gladbach
MicroAmp® Fast Optical 96-Well Reaction Plate	ThermoFisher, Life Technologies, Berlin
Neubauer Counting Chamber (0.1-0.0025 mm^2)	Hecht-Assistent, Sondheim
Pasteur Glass Pipettes 1 mL	ThermoFisher, Life Technologies, Berlin
Salivetten	Sarstedt, Nümbrecht
Seahorse Calibration Plate	Agilent, Waldbronn
Seahorse Cell Culture Plate	Agilent, Waldbronn
Single Use Pipettes (5 mL, 10 mL, 25 mL)	Becton Dickinson, Heidelberg
Sterile Filter (0.22 µm)	Millipore, Schwalbach
Syringe 30 mL	Becton Dickinson, Heidelberg

2.2.3 Reagents and Chemicals

All chemicals and reagents used are listed in Table 8.

Table 8: Reagents, chemicals and analysis kits.

Anti-Mouse Ig, κ/Negative Control Compensation Particles Set	Becton Dickinson, Heidelberg, Germany
a-CD28	eBioscience, ThermoFisher, Berlin
Biocoll	Merck, Biochrom, Darmstadt, Germany
Bovine Serum Albumin	Serva, Heidelberg
Cortisol Detection Kit	IBL, Hamburg
DMSO	AppliChem GmbH, Darmstadt
FCCP	Agilent, Waldbronn
FCS S0115/1318D	Merck, Biochrom, Darmstadt, Germany
Glucose	Sigma, Eisenhofen
L-Glutamine	ThermoFisher, Life Technologies, Berlin
MACS BSA stock solution	Miltenyi, Bergisch Gladbach
Auto MACS Rinsing Solution	MACS, Miltenyi Biotech, Bergisch Gladbach
MACS BSA Stock Solution	MACS, Miltenyi Biotech, Bergisch Gladbach
Oligomycin	Agilent, Waldbronn
OKT, a-CD3 clone	eBioscience, ThermoFisher, Berlin
4% Paraformaldehyde solution in 1x PBS	Santa Cruz Biotechnology, Heidelberg
1x PBS	Gibco, Life Techonogies, Berlin, Germany
Perm/Wash, Cytofix/Cytoperm	Becton Dickinson, Heidelberg
Rotenone antimycin A	Agilent, Waldbronn
RPMI 1640 Medium, GlutaMAX™ Supplement	GIBCO, Life Technologies, Berlin, Germany
Seahorse XF Base Medium	Agilent, Waldbronn
Sodium Azide	Sigma, Eisenhofen
Sodium Pyruvate	ThermoFisher, Life Technologies, Berlin
Trypan Blue	Sigma, Eisenhofen

2.2.4 Cell Culture Media

Cell culture medium	**Seahorse mitochondrial stress test medium**
RPMI 1640 Medium, GlutaMAX™	Seahorse XF Base Medium
supplement:	110.4 g/mol Sodium Pyruvate
10 % FCS	10 mM Glucose
	2 mM L- Glutamine

Cell freezing medium

RPMI 1640 Medium, GlutaMAX™
supplement:
25 % FCS
10 % DMSO

2.2.5 Materials for RNA isolation and cDNA Synthesis

Materials and kits used for ribonucleic acid (RNA) isolation and complementary deoxy-ribonucleic acid (cDNA) synthesis are listed in Table 9.

Table 9: RNA isolation and cDNA synthesis kits and reagents.

RNAlater	ThermoFisher, Life Technologies, Berlin
RNeasy Plus Universal Mini Kit	Qiagen, Hilden
cDNA synthesis kit	ThermoFisher, Life Technologies, Berlin

2.2.6 Materials and Reagents for qRT-PCR Analyses

All materials and reagents for quantitative real time-polymerase chain reaction (qRT-PCR) analyses were supplied by ThermoFisher Scientific, Life Technologies, Berlin (Table 10).

Table 10: qRT-PCR materials and reagents. [a]

TaqMan Gene Expression Kit		
TaqMan Gene Expression Master Mix		
TaqMan Gene Probe	**Gene Name**	**Taq Man ID**
GLUT1	SLC2A1	Hs00892681_m1
TNF alpha g1	Tumor Necrosis Factor	Hs01113624_g1
CPT1a	CPT1a	Hs00912671_m1
NFκB1	Nuclear Factor kappa B subunit 1	Hs00765730_m1
NFκB3 p65 subunit	RELA proto-oncogene, NFκB-subunit	Hs00153294_m1
GR	NR3C1	Hs00353740_m1
GILZ	TSC22 domain family member 3	Hs00608272_m1
TBP	TATA Box Binding Protein	Hs00427620_m1
Importin 8	IPO8	Hs00183533_m1

[a] all supplied by ThermoFisher, Life Technologies, Berlin.

The *CPT1a* gene probe binds five different reference sequences [123] including the one detected by the flow cytometry CPT1a antibody. For *NFκB expression* analysis, two subunits were chosen due to the complexity of the functional protein. *NFκB3* (subunit p65) most commonly forms the functional dimer of the final protein along with *NFκB 1* (subunit p50).

2.2.7 Buffer and Stock Solutions for Magnetically Activated Cell Sorting and Flow Cytometry Analyses

MACS buffer

5 % MACS BSA Stock Solution

in Auto MACS Rinsing Solution

Flow cytometry buffer

1 x PBS

0.5 % BSA

0.02 % Sodium Azide

2.2.8 Magnetically Activated Cell Sorting and Flow Cytometry Antibodies

A full list of antibodies used for magnetically activated cell sorting (MACS) and flow cytometry analyses is provided in Table 11 and Table 12 respectively.

Table 11: List of antibodies used for Magnetically Activated Cell Sorting (MACS) and flow cytometry.

MACS antibodies

antigen	linked to	clone	concentration	company
CD4	Micro beads	n/a	1:5	Miltenyi, Biotech GmbH, Bergisch Gladbach
CD8	Micro beads	n/a	1:5	

Flow cytometry analysis antibodies

antigen	linked to	clone	concentration	company
CPT1a	AlexaFluor®488	8F6AE9	1:100	Abcam, Cambridge, UK
IgG2b	AlexaFluor®488	7E10G10	1:100	Abcam, Cambridge, UK
CD279 (PD-1)	PE	EH12.2H7	1:40	Biotechne, Wiesbaden
CD127 (IL-7Rα)	APC	A019D5	1:50	Biolegend, London, UK
CD16	APC	3G8	1:500	Biolegend, London, UK
CD194 (CCR4)	APC	L291H4	1:25	Biolegend, London, UK
CD197 (CCR7)	APC	G043H7	1:20	Biolegend, London, UK
CD14	Brilliant Violet 421™	HCD14	1:500	Biolegend, London, UK
CD183 (CXCR3)	Brilliant Violet 421™	G025H7	1:100	Biolegend, London, UK
CD25 (IL-2Rα)	Brilliant Violet 421™	M-A251	1:100	Biolegend, London, UK
CD3	Brilliant Violet 421™	UCHT1	1:200	Biolegend, London, UK
CD3	Brilliant Violet 510™	UCHT1	1:25	Biolegend, London, UK
CD8a	Brilliant Violet 510™	RPA-T8	1:25	Biolegend, London, UK
CD196 (CCR6)	PE/Cy7	G034E3	1:50	Biolegend, London, UK
CD20	PE/Cy7	2H7	1:500	Biolegend, London, UK
CD45RA	PE/Cy7	HI100	1:200	Biolegend, London, UK
CD4	PerCP/Cy5.5	PRA-T4	1:50	Biolegend, London, UK
CD56 (NCAM)	PerCP/Cy5.5	HCD56	1:200	Biolegend, London, UK
Live/dead marker	ZombieNIR™ Fixable Viability Kit	n/a	1: 1000	Biolegend, London, UK

Table 12: Four antibody panels for cell phenotyping, PD-1 and CPT1a analysis.

Panel 1: memory T cell subtypes

fluorochrome	conjugate
Alexa Fluor 488	CPT1a
PE	PD-1
PerCP-Cy5.5	CD4
PE-Cy7	CD45RA
APC	CCR7
Zombie NIR™	Live/dead differentiation
BV421™	CD3
BV510™	CD8

Panel 2: effector T cell subtypes

fluorochrome	conjugate
Alexa Fluor 488	CPT1a
PE	PD-1
PerCP-Cy5.5	CD4
PE-Cy7	CCR6
APC	CCR4
Zombie NIR™	Live/dead differentiation
BV421™	CXCR3
BV510™	CD8

Panel 3: regulatory T cells

fluorochrome	conjugate
Alexa Fluor 488	CPT1a
PE	PD-1
PerCP-Cy5.5	CD4
PE-Cy7	CD45RA
APC	CD127
Zombie NIR™	Live/dead differentiation
BV421™	CD25
BV510™	CD3

Panel 4: B cells, NK cells, monocytes

fluorochrome	conjugate
Alexa Fluor 488	CPT1a
PE	PD-1
PerCP-Cy5.5	CD56
PE-Cy7	CD20
APC	CD16
Zombie NIR™	Live/dead differentiation
BV421™	CD14
BV510™	CD3

2.2.9 Software used for analyses

Table 13 lists all software used for data analyses and corresponding company names.

Table 13: Software.

software	**company**
FACSDiva version 6.1.2	Becton Dickinson, Heidelberg, Germany
FlowJo® 10.3	FlowJo, LLC, Ashland, OR, USA
GraphPad Prism 7	GraphPadSoftware Inc., La Jolla, CA, USA
Microsoft Office 2016	Microsoft, Berlin, Germany
SPSS 23	IBM Inc., Berlin, Germany
Step One Plus™	ThermoFisher, Life Technologies, Berlin, Germany
Wave Desktop 2.3	Agilent, Waldbronn, Germany

2.3 Methods

2.3.1 Cellular Biology

2.3.1.1 Cryo-preservation of Peripheral Blood Mononuclear Cells (PBMC)

Peripheral Blood Mononuclear Cells (PBMCs) were separated from about 80 mL of fresh whole blood by density gradient centrifugation (Biocoll Separating Solution, Biochrom GmbH). The time between blood draw and PBMC isolation did not exceed 30 minutes for all MS patients and HC participants.

To obtain isolated PBMCs, whole blood was diluted 1:2 with 1xPBS (phosphate buffered saline) (room temperature (RT)) and carefully layered on top of 15 mL of Biocoll Separating Solution (Merck, Biochrom) (RT). This preparation was centrifuged for 30 min (RT) at 300xg with the centrifuge break off. The PBMC monolayer was distracted from the separated blood contents and washed two times in 1xPBS (4°C). PBMCs were added to RPMI 1640 GlutaMAX™ medium (GIBCO, Life Technologies) supplemented with 25% FCS (Merck, Biochrome) and 10% DMSO (AppliChem GmbH). Aliquots of $1x10^7$ cells/cryo tube were slowly frozen to -80°C at 10°C per hour intervals in a Mr. Frosty container (Nalgene®). After 20-30 hours, the frozen aliquots were transferred into liquid nitrogen for long time storage.

2.3.1.2 Thawing of PBMCs

PBMCs in cryo tubes were taken out of the liquid nitrogen container and placed on ice. Cells were thawed quickly to 37°C and subsequently resuspended in 45 mL of 37°C RPMI medium with GlutaMAX™ supplemented with 10% FCS and centrifuged at 300xg for 5 minutes. The supernatant was taken off and the cells were washed in 1x PBS (RT) and centrifuged at 300xg for 5 minutes. The supernatant was taken off again and cells were prepared according to protocols.

2.3.1.3 MACS Positive Selection

$CD4^+$ and $CD8^+$ T cells from MS patients and HC participants were purified from PBMCs using magnetic bead positive selection (Miltenyi). All steps were performed with reagents and the centrifuge at 4°C.

PBMCs were thawed according to protocol (see 2.3.1.2). After the last washing step, the supernatant was taken off and 80 µL of MACS buffer and 20 µL CD4 positive T cell selection MACS antibody (Miltenyi) were added per $5x10^6$ cells. The cell suspension was incubated at 4°C in the dark for 15 minutes and subsequently washed with 5 mL MACS buffer (300xg, 5 min.). The supernatant was taken off and the cells were resuspended in 3 mL MACS buffer. An LS column (Miltenyi) was placed into the Quadro MACS magnet and rinsed three times with 3 mL MACS rinsing buffer (Miltenyi). Subsequently, the 3 mL cell suspension was applied onto the column. Here, the magnetically labeled $CD4^+$ T cells were retained inside the column and the non-labeled cells (negative fraction) were washed out into a 15 mL falcon tube. To retrieve the magnetically labeled cells, the column was placed outside the magnet into a new 15 mL falcon tube and rinsed with 5 mL MACS buffer. The cells were centrifuged at 300xg for 5 min. and the supernatant was taken off. Following the $CD4^+$ T cell selection, $CD8^+$ T cells were separated from the negative fraction using the same protocol and the CD8 positive selection MACS antibody (both Miltenyi).

Subsequently, three fractions were obtained: $CD4^+$ T cells, $CD8^+$ T cells and a negative fraction containing PBMCs lacking the separated $CD4^+$ and $CD8^+$ T cells - the "non-$CD4^+$/$CD8^+$ T cell" fraction. The non-$CD4^+$/$CD8^+$ T cell fraction mainly compromises B cells, NK cells and monocytes. After the MACS separation, all fractions were taken up in RPMI medium with GlutaMAX™ supplemented with 10% FCS.

2.3.1.4 Cell Culture of Sorted PBMC subtypes

Following MACS separation (see 2.3.1.3), the three populations, $CD4^+$ T cells, $CD8^+$ T cells and non-$CD4^+$/$CD8^+$ T cells, were seeded at a density of $2x10^6$ cells/well in a six well plate in RPMI medium with GlutaMAX™ and 10% FCS. The cells were incubated for 2 h in a 5% CO_2 incubator at 37°C.

2.3.2 In vitro Metabolic Analyses using the Seahorse XF^{e}96 Analyzer

2.3.2.1 Establishing the Mitochondrial Stress Test Analysis Protocol

In order to contribute valid data, the Mitochondrial Stress Test Analyses using the Seahorse XF^e 96 analyzer had to be established for human immune cell bioenergetic analyses. According to the manufacturer's instructions (Agilent), the following tests were performed: titration of optimal FCCP, oligomycin and rotenone with antimycin A concentrations as well as the required cell number per well. The following concentrations were determined as optimal based on the pre-analyses assays: FCCP: 2 µM, oligomycin 1 µM, rotenone with antimycine A: 0.5 µM. The cryopreserved PBMCs from a healthy donor were thawed 10 days after blood draw for the assay analysis (see 2.3.1 for methods). Cell density tests revealed 4x10^5 cells/well giving optimal OCR values (Figure 6). Data was analyzed using the manufacturer's provided Mito Stress Test Kit Generator V2. Due to limited PBMC material from MS patients and HC participants, no more than 4x10^5 cells/well could be used.

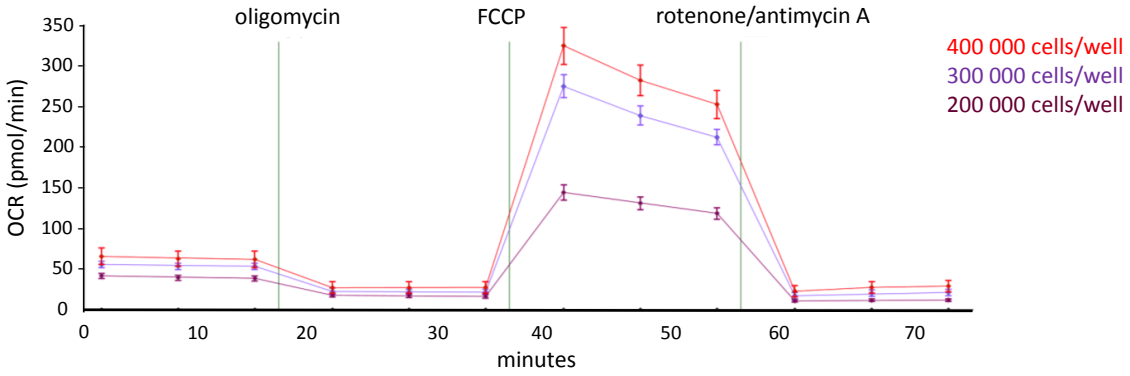

Figure 6: Seahorse XF^e 96 Analyzer assay test analyses for cell density determination shows increases in OCR with increasing PBMC numbers. The graphs show Peripheral Blood Mononuclear Cells (PBMCs) from a healthy donor. The cells were cryo preserved after blood draw and thawed after 10 days for Seahorse Mitochondrial Stress Test Kit assay analysis. The data was generated in real-time under basal conditions, in response to oligomycin, FCCP and Rotenone and Antimycin A using a Seahorse XF^e 96 Analyzer (Agilent). Three different cell densities were analyzed: 2x10^5 cells/well, 3x10^5 cells/well and 4x10^5 cells/well. Mean and SEM calculated per condition for each time point. OCR: oxygen consumption rate, FCCP: Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone.

In this thesis, the main aim was to analyze $CD4^+$ and $CD8^+$ T cells. $CD4^+$ and $CD8^+$ T cells are non-adherent, therefore, it was tested whether the cells are detaching from the culture plate during mixing times in the Seahorse analyzer. This would lead to inaccurate readouts, because energy consumption of detaching cells floating in the medium would not be measured. Cellular adherence was examined using poly-D-lysine (PDL) in the Seahorse cell culture plates according to the manufacturer's instructions (Agilent). PBMCs from a healthy donor were thawed and $CD4^+$ and $CD8^+$ T cells were purified using MACS. Furthermore, a non-$CD4^+$/$CD8^+$ T cell fraction lacking $CD4^+$ and $CD8^+$ T cells was obtained (see 2.3.1.3 MACS Positive Selection). The three cell populations were incubated at 37°C in a 5% CO_2 incubator for 2 h at $2x10^6$ cells/well in a six well plate (see for 2.3.1 methods). Subsequently, the cells were washed with Seahorse mitochondrial stress test medium (5 min. at 300xg) and seeded at $4x10^5$ cells/well into the according Seahorse cell culture plates previously coated with PDL or non-coated.

No differences in bioenergetic readouts were observed between PDL treated versus PDL non-treated Seahorse cell culture plates (at least 5 wells/assay condition were analyzed) (Figure 7). Therefore, using PDL to attach T cells to the Seahorse cell culture plate was not necessary for T cell analyses. Furthermore, with respect to possible activation of immune cells by PDL, it was moreover of advantage not to use PDL in order to get valid assay results.

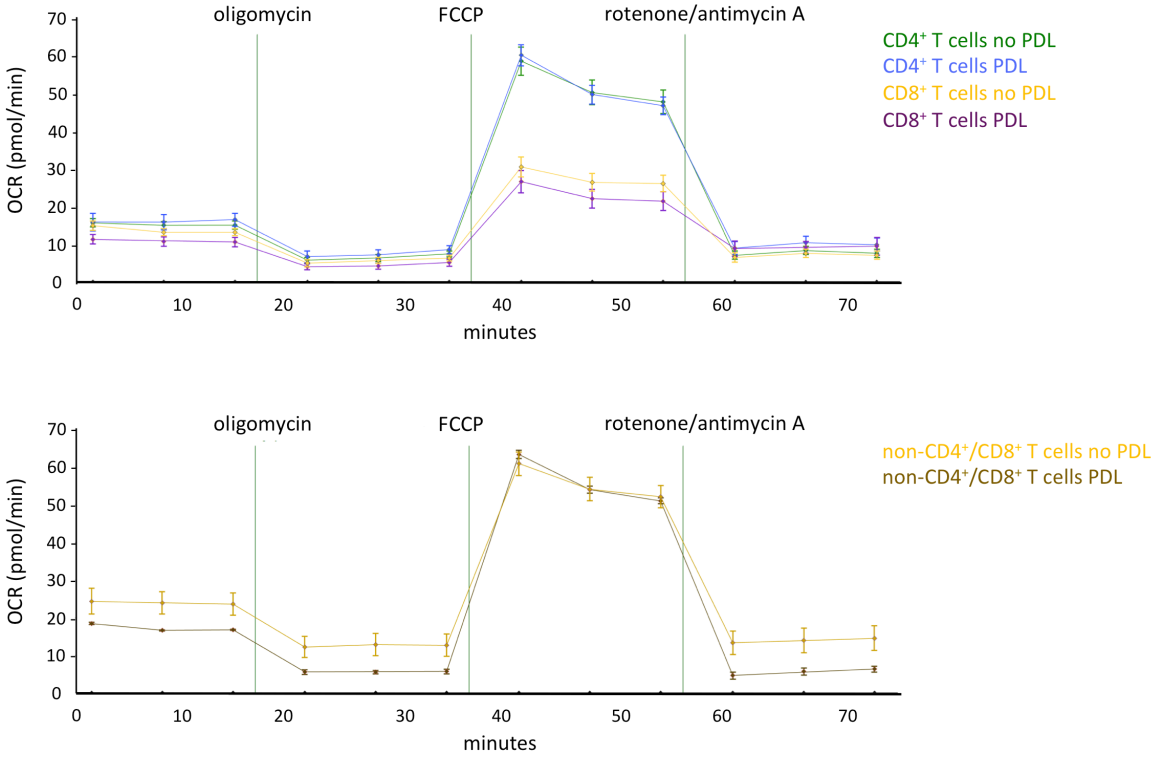

Figure 7: Seahorse XF^e 96 Analyzer metabolic assay cellular adherence testing shows no difference in OCR measurements with or without PDL plate coating. The graphs shows magnetically purified CD4$^+$ T cells, CD8$^+$ T cells and non-CD4+/CD8+ T cells (PBMCs lacking CD4$^+$ and CD8$^+$ T cells) from a healthy donor incubated and measured with or without previous PDL plate coating. Cryopreserved PBMCs were thawed after 90 days for Seahorse Mitochondrial Stress Test Kit assay analysis. The data was generated in real-time under basal conditions, in response to oligomycin, FCCP and Rotenone and Antimycin A using a Seahorse XF^e 96 Analyzer (Agilent). Cell density: 4x10^5 cells/well. Mean and SEM calculated per condition for each time point. PDL: Poly-D-Lysine, OCR: oxygen consumption rate, FCCP: Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone.

Furthermore, previous studies of Keane *et al.* [124] showed that human PBMCs display decreasing bioenergetics over time in cryo-preservation. Keane and colleagues showed that after 50 days of storage, bioenergetic levels stabilized and did not decrease further. To ensure equal treatment and exclusion of bioenergetic effects caused by freezing duration to the highest extent possible, it was decided that all MS patients' and HC participants' PBMC samples were analyzed no earlier than 50 days after liquid nitrogen storage. Additionally, it was ensured that there was no correlation between MS patients' and HC participants' PBMC freezing duration and metabolic, genetic or FACS analyses.

Finally, to test PBMC activation and compare it to freshly thawed PBMCs (all from the same healthy donor), α-CD3 and α-CD28 stimulation assays were performed.

Hence, after thawing (see 2.3.1), $2x10^6$ PBMCs/well were seeded into a six well plate and stimulated with 1 mg/mL α-CD3 (clone OKT) and 1 µg/mL α-CD28 (both eBioscience, ThermoFisher) for 2 h, 22 h or left unstimulated. These three conditions were compared to freshly thawed PBMCs. It was observed, that freshly thawed and analyzed PBMCs had higher metabolic rates compared to PBMCs that were stimulated for 2 h, 22 h or not stimulated at all during the 22 h incubation (see Figure 8).

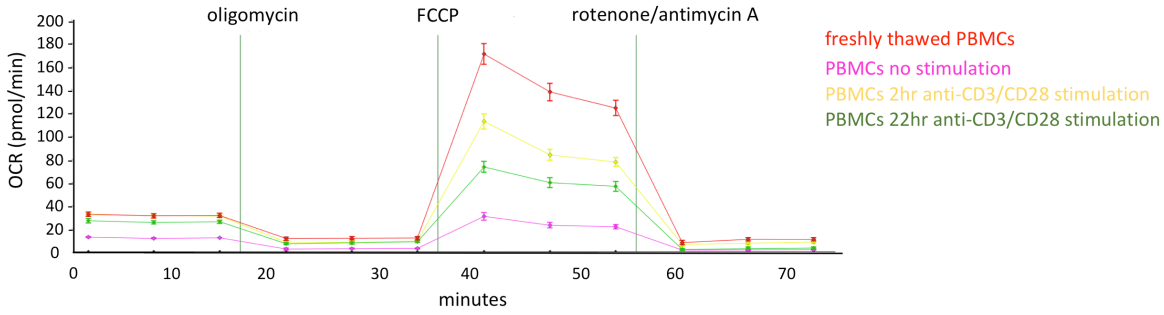

Figure 8: Seahorse XF^e 96 Analyzer metabolic assay PBMC stimulation testing with α-CD3 and α-CD28. The graphs show PBMCs from a healthy donor. Cryopreserved PBMCs were thawed after 225 days for assay analysis. Four assay conditions were analyzed: PBMCs incubated for 22 h without stimulation, PBMCs stimulated with α-CD3 and α-CD28 for 22 h, PBMCs stimulated with α-CD3 and α-CD28 for 2 h and freshly thawed PBMCs without a stimulation. Assay used: Seahorse Mitochondrial Stress Test Kit assay. Cell density: $4x10^5$ cells/well. The data was generated in real-time under basal conditions, in response to oligomycin, FCCP and Rotenone and Antimycin A using a Seahorse XF^e 96 Analyzer (Agilent). Mean and SEM calculated per condition for each time point. PBMC: Peripheral Blood Mononuclear Cell, OCR: oxygen consumption rate, FCCP: Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone.

The final Seahorse XF^e 96 Analyzer protocol was established from all extensive pre-assay experiments and analyses (described in detail in 2.3.2).

Analyzing samples from a cross-sectional clinical study recruiting MS patients and closely matched HC participants simultaneously at two different medical centers within a time frame of two years, the laboratory analyses and study organization and management were controlled for and planned as best as possible.

2.3.2.2 Metabolic Analyses of $CD4^+$ T cells, $CD8^+$ T cells and non-$CD4^+$/$CD8^+$ T cells

Using the Seahorse Mitochondrial Stress Test Kit assay, $CD4^+$ T cells, $CD8^+$ T cells and non-CD4+/CD8+ T cells from MS patients and matched HC participants were analyzed in the Seahorse XF^e 96 analyzer. An MS patient and the accordingly matched HC participant pair was analyzed on the same Seahorse cell culture plate to ensure comparability and avoid inter-assay variance. Based on the manufacturer's recommendations, samples were analyzed in at least triplicates and the mean was calculated to ensure data accuracy and omit inter-well differences. All samples were frozen for at least 50 days prior to thawing.
After PBMC purification, $CD4^+$ T cells, $CD8^+$ T cells and non-$CD4^+$/$CD8^+$ T cells were rested for two hours. Subsequently, the cells were harvested and washed with Seahorse Mitochondrial Stress Test Kit medium (300xg, 5 min.). The cells were seeded at a density of $4x10^5$ cells per well into a Seahorse cell culture plate and incubated wrapped in a plastic bag at 37 °C in a non-CO_2 incubator for 30 minutes. The sensor cartridge (Agilent Technologies) was incubated for 4 h with calibration buffer (Agilent Technologies) wrapped in a plastic bag at 37 °C in a non-CO_2 incubator. Prior to putting the calibration plate into the Seahorse analyzer, the appropriate ports were filled with reagents: 20 µL of 2 µM FCCP in port A, 22 µL of 1 µM oligomycin in port B, 25 µL of 0.5 µM rotenone with antimycin A in port C and 27 µL of Seahorse Mitochondrial Stress Test Kit medium in port D (all Agilent Technologies). All reagents were prepared with Seahorse Mitochondrial Stress Test Kit medium. To prevent vaporization of the reagents in the ports, the sensor cartridge was immediately placed into the analyzer for a 15 min. calibration, which is required by the machine and follows the company's instructions. Finally, the cell culture plate was added to the Seahorse analyzer and the analysis started using the protocol provided in Table 14.

Table 14: Seahorse XF^e 96 analyzer mitochondrial stress test kit analysis protocol.

equilibration 12 minutes
baseline readings (loop 3 times)
mix 3 min – wait – 3 min measure
inject port A oligomycin (loop 3 times):
mix 3 min – wait – 3 min measure
inject Port B FCCP (loop 3 times):
mix 3 min – wait – 3 min measure
inject Port C rotenone and antimycin A (loop 3 times):
mix 3 min – wait – 3 min measure
program ends.

2.3.2.3 Evaluation of MS Patient and HC Participant Samples

The Seahorse XF^e 96 Analyzer measures the changes in oxygen consumption rate (OCR, pmols/min) and extracellular acidification rate (ECAR, mpH/min) in the medium under real-time conditions. Therefore, direct conclusions about mitochondrial aerobic respiration (OCR) and the changes in pH in the medium (ECAR) can be drawn. The acidification rate is the result of changes in pH mainly due to cellular lactate production and CO_2 release from the cells. The cells were analyzed in real-time under four different conditions: 1) basal respiration without any reagents in the Seahorse Medium, respiration in response to 2) oligomycin, 3) FCCP and 4) rotenone and antimycin A. The values measured by the Seahorse XF^e 96 Analyzer were analyzed using the Wave software and Seahorse XF^e 96 Mito Stress Test Generator V2 (both Agilent, Waldbronn). OCR (pmoles/min.) values for basal respiration (BR), spare respiratory capacity (SRC), maximal respiration (MR), proton leakage, ATP production and non-mitochondrial respiration (NMR) were obtained. The equations the Seahorse XF^e 96 Mito Stress Test Generator V2 (Agilent, Waldbronn) used to determine each parameter are listed in Table 15.

Table 15: Seahorse Analyzer Parameter Calculations for OCR and ECAR using the mitochondrial stress test kit. (table adapted from [125])

parameter value	equation
Non-mitochondrial oxygen consumption	Minimum rate measurement after rotenone/antimycin A injection
Basal respiration	(last rate measurement before first injection)-(NMR)
Maximal respiration	(maximum rate measurement after FCCP injection)-(NMR)
H^+ (proton) leak	(minimum rate measurement after oligomycin injection)-(NMR)
ATP production	(last rate measurement before oligomycin injection)-(minimum rate measurement after oligomycin injection)
Spare respiratory capacity	(MR)-(BR)
Basal ECAR	last rate measurement before first injection
Maximal ECAR	maximum rate measurement after oligomycin injection

NMR: Non-mitochondrial respiration, BR: basal respiration, MR: maximal respiration, PL: proton leak, ATP: adenosine triphosphate, SRC: spare respiratory capacity, ECAR: Extracellular Acidification Rate.

Using the mitochondrial stress test assay for the Seahorse XF^e 96 analyzer, basal extracellular acidification rate (ECAR) and maximal ECAR after oligomycin injection can be evaluated (Table 15). Changes in mpH/min. after FCCP injection include changes in the medium resulting from mitochondrial respiration and other bioenergetic pathways in the cells (e.g. TCA cycle, pentose phosphate pathway), which influence the pH of the medium. Therefore, only basal ECAR (last rate measurement before oligomycin injection) and maximal ECAR (highest value after oligomycin injection) were analyzed. These values in pH changes in the medium provide data for basal cellular glycolysis rates in unstimulated cells and maximal glycolysis rates during mitochondrial ETC complex V inhibition.

Due to differing outcomes in cell number from MS patients after density gradient centrifugation PBMC isolation and because of differences in cell viability potentially caused by MS medication, 24 MS patient-HC participant pairs out of the 31 pairs included in the study analyses (Table 16) could be analyzed.

The real-time measurement of cell viability can cause readouts that can't be used for analyses, mainly due to cell viability during the assay run or possible air bubbles in the well interfering with the Seahorse Analyzer system. If, under the assay conditions, cell

viability suffered or, by definition, $CD8^+$ T cell purification yielded lower outcome and could not be analyzed, the data was excluded resulting in the differing numbers of pairs analyzed (n-numbers). However, at least three wells per cell population were used for analyses in order to ensure data and statistical accuracy as well as to omit inter-well differences. If less than three wells were analyzable, the data was excluded. For three or more wells per cell population, the mean was calculated and used for data analyses. Exemplarily: for MS patients' $CD8^+$ T cells on average 5.5 wells (mean, SD 2.3) could be used for analyses. For HC participants' $CD8^+$ T cells on average 5.6 wells (mean, SD 2.2) were used for analyses. There was no significant difference in the number of wells analyzed from MS patients and HC participants for $CD4^+$ T cells, $CD8^+$ T cells and non-$CD4^+$/$CD8^+$ T cells (all $p \geq 0.5$).

2.3.3 Flow Cytometry Analyses

2.3.3.1 Intracellular and Extracellular Staining

PBMCs from an MS patient and correspondingly matched HC participant were always analyzed in parallel in the flow cytometer. The cryo preserved PBMC vials used were all prepared from the one blood draw at the study visit (2.3.1), which ensures comparability of the results between the different experiments. The antibodies are listed in Table 11, the corresponding antibody panels used are listed in

Table 12 and Figure 15 give an overview of the final cell phenotypes analyzed. The antibody panels are based on a publication by Maecker *et al.* 2012 [126] and were modified to incorporate CPT1a and PD-1 analyses.

For flow cytometry antibody staining, one cryo tube per MS patient and HC participant with $1x10^7$ cells was thawed. The cells were aliquoted in four 5 mL flow cytometry tubes for each panel. The tubes were incubated in the dark at RT for 15 minutes with the live/dead marker Zombie NIR in 50 µL 1xPBS. For staining panel 1 (Table 12), the CCR7-APC antibody was also added to the solution. Subsequently, 50 µL of the extracellular antibody mix of each panel (Table 12) were added for an additional 15 min. incubation (RT, in the dark). The cells were then washed in 1 mL FACS buffer (300xg, 5 min.).

For intracellular staining, 100 µL cytofix/cytoperm (Becton Dickinson) were added and cells were incubated for 15 min. (RT, in the dark). The cells were washed in 1 mL perm/wash (Becton Dickinson) (300xg, 5 min.). Anti-CPT1a-AlexaFluor®488 was diluted in 100 µL perm/wash and incubated with the cells for 30 minutes at RT in the dark. For isotype control analyses, the corresponding isotype was used. The cells were washed in 1 mL perm/wash (300xg, 5 min.). 300 µL FASC buffer was added and the cells were analyzed within 2 hours.

2.3.3.2 Titration of Antibodies

Antibodies used for all four multi-color antibody panels in flow cytometry analyses (Table 11) were titrated to their optimal concentration (Intracellular and Extracellular Staining).

2.3.3.3 Gating Strategy

Flow cytometry gates were set and cell populations determined based on the antibody panels used (Table 12). Figure 9 through Figure 13 display the gating strategy for each antibody panel. Figure 15 summarizes the final cell phenotypes based on the antibody panels used.

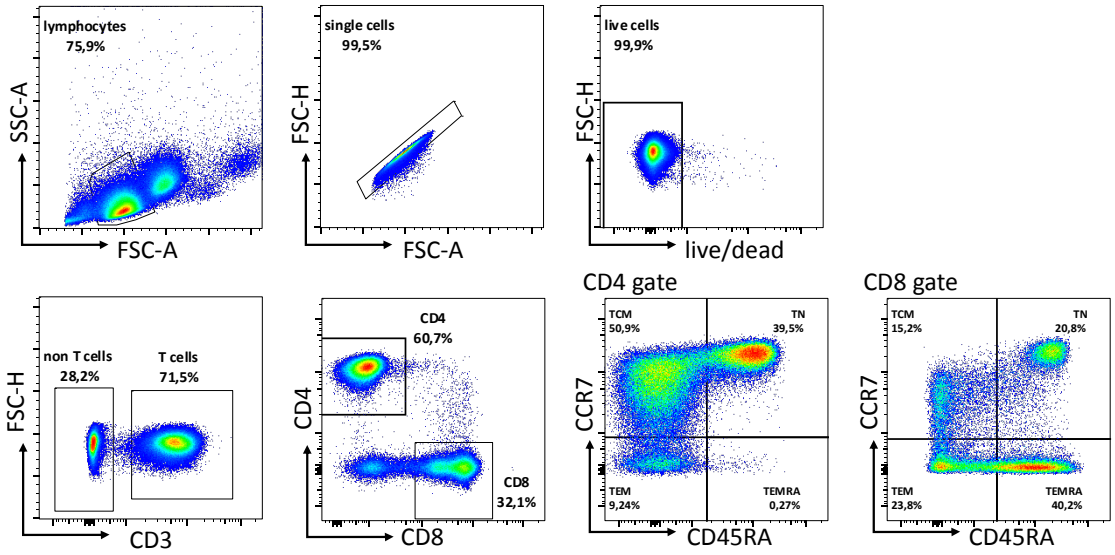

Figure 9: Gating strategy for panel 1 of phenotyping analyses to determine memory subtypes in CD4$^+$ and CD8$^+$ T cells. Flow cytometry analyses of cryopreserved PBMCs from MS patients and HC participants. Lymphocytes were identified based on size using forward scatter (FSC) and side scatter (SSC). Doublets were excluded based on FSC-H (height) and FSC-A (area) followed by dead cell exclusion. T cells and non-T cells were discriminated based on CD3 surface expression. Further

determination of T cells was distinguished by CD4 and CD8 surface expression. T cell memory subsets were discriminated based on CCR7 and CD45RA surface expression: TCM: CCR7$^+$ CD45RA$^-$, naïve: CCR7$^+$ CD45RA$^+$, TEM: CCR7$^-$ CD45RA$^-$, TEMRA: CCR7$^-$ CD45RA$^+$. TCM: central memory T cell, TEM: effector memory T cell, TEMRA: terminally differentiated effector memory cells re-expressing CD45RA.

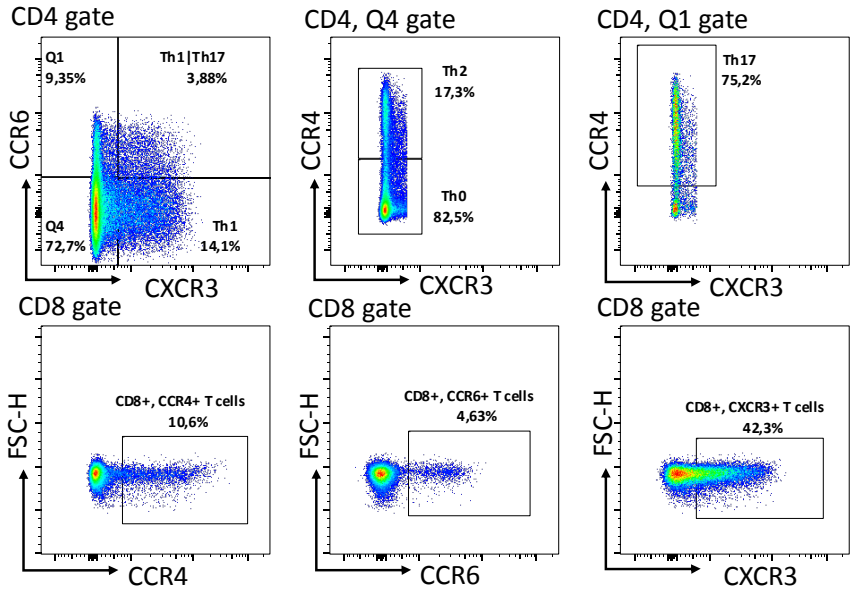

Figure 10: Gating strategy for panel 2 of phenotyping analyses to determine CD4$^+$ helper T cells and CD8$^+$ T cell subtypes. Flow cytometry analyses of cryopreserved PBMCs from MS patients and HC participants. Cells were gated on single cell live lymphocytes as shown in Figure 9: lymphocytes were identified based on size using forward scatter (FSC) and side scatter (SSC). Doublets were excluded based on FSC-H (height) and FSC-A (area) followed by dead cell exclusion. T cells and non-T cells were discriminated based on CD3 surface expression. Further determination of T cells was distinguished by CD4 and CD8 surface expression. CD4$^+$ helper T cell subsets were discriminated based on the differential expression of CCR4, CCR6 and CXCR3. Th1: CCR6$^-$ CXCR3$^+$, Th1/Th17: CCR6$^+$ CXCR3$^+$, Th2: CCR6$^-$ CXCR3$^-$ CCR4$^+$, Th0: CCR6$^-$ CXCR3$^-$ CCR4$^-$, Th17: CCR6$^-$ CXCR3$^-$ CCR4$^+$. The expression of CCR4, CCR6 and CXCR3 was further analyzed on CD8$^+$ T cells. CCR: CC chemokine receptor, CXCR3: CXC chemokine receptor 3.

CD4$^+$ CD25$^-$ CD127$^+$ conventional T cells were discriminated from T regs by a boolean gate including all CD4$^+$ T cells but CD25$^+$ and CD127^{low} (IL7-Rα) T regs (Figure 11). In addition to defining cell populations using CD127, the median fluorescent intensities (MFI) of IL7-Rα and IL2-Rα were analyzed in conventional T cells of MS patients and HC participants.

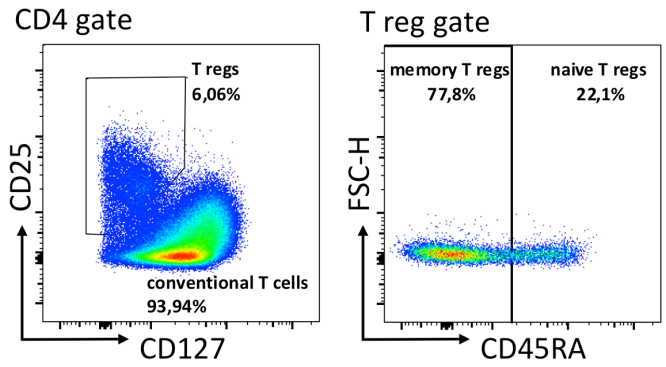

Figure 11: Gating strategy for panel 3 of phenotyping analyses to determine regulatory T cells in CD4$^+$ T cells. Flow cytometry analyses of cryopreserved PBMCs from MS patients and HC participants. Cells were gated on single cell live lymphocytes as shown in Figure 9: lymphocytes were identified based on size using forward scatter (FSC) and side scatter (SSC). Doublets were excluded based on FSC-H (height) and FSC-A (area) followed by dead cell exclusion. T cells and non-T cells were discriminated based on CD3 surface expression. helper T cells were distinguished by CD4 surface expression. T regs were then discriminated based on CD25/CD127^{low} surface expression and further divided into naïve and memory T regs based on CD45RA expression. CD4$^+$ CD25$^-$ CD127$^+$ conventional T cells were defined by a boolean gate including all CD4$^+$ T cells but T regs. T reg: regulatory T cell.

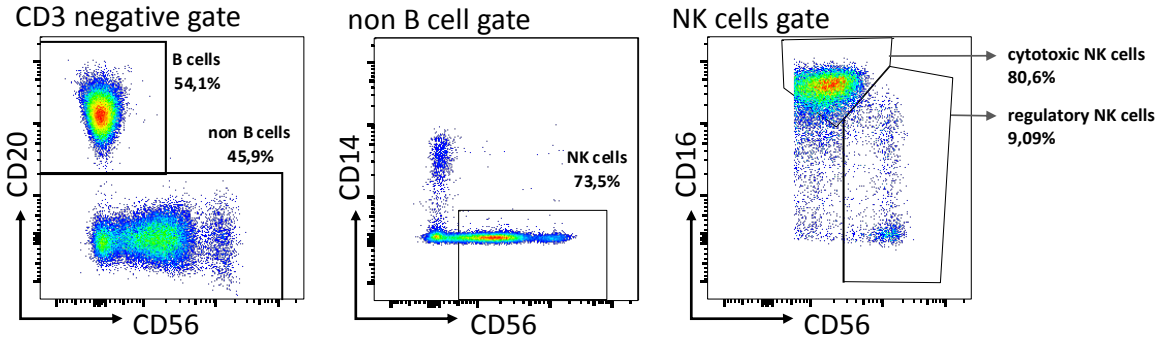

Figure 12: Gating strategy for panel 4 of phenotyping analyses to determine B cells and NK cells. Flow cytometry analyses of cryopreserved PBMCs from MS patients and HC participants. Cells were gated on single cell live lymphocytes as shown in Figure 9: Lymphocytes were identified based on size using forward scatter (FSC) and side scatter (SSC). Doublets were excluded based on FSC-H (height) and FSC-A (area) followed by dead cell exclusion. T cells and non-T cells were discriminated based on CD3 surface expression. CD3$^-$ non-T cells were further discriminated for CD20, CD56 and CD16 expression. B cells: CD20$^+$, cytotoxic NK cells: CD16$^+$ CD56$^-$, regulatory NK cells: CD16$^{int./-}$ CD56$^+$. NK cell: natural killer cell.

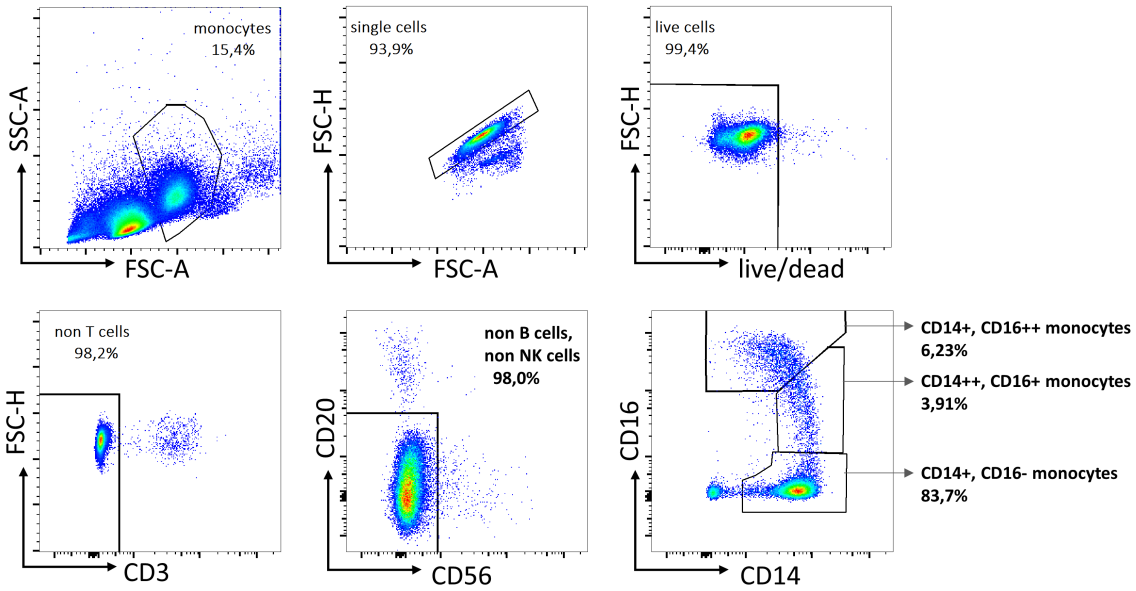

Figure 13: Gating strategy for panel 4 of phenotyping analyses to determine monocyte subpopulations. Flow cytometry analyses of cryopreserved PBMCs from MS patients and HC participants. Monocytes were identified based on forward scatter (FSC) and side scatter (SSC) properties. Doublets were excluded based on FSC-H (height) and FSC-A (area) followed by dead cell exclusion. T cells and non-T cells were discriminated based on CD3 surface expression. Contaminating B cells and NK cells were excluded based on CD20 and CD56 expression. Monocyte subsets were identified by the differential expression of CD14 and CD16: $CD14^{+}$ $CD16^{++}$ non-classical monocytes, $CD14^{++}$ $CD16^{+}$ intermediate monocytes, $CD14^{++}$ $CD16^{-}$ classical monocytes. NK cell: natural killer cell.

Figure 14 shows a representative PD-1 staining and a CPT1a antibody staining compared to its isotype control. $CD4^{+}$ naïve T cells (full gating shown in Figure 9) were set as a negative reference (Figure 14 B) to determine PD-1 positive T cell populations. An exemplary histogram is shown with $CD4^{+}$ effector memory T cells. For analysis, the median fluorescent intensity (MFI) of the PD-1 negative reference population of interest was subtracted from the MFI of the positive population of interest.

For the analysis of CPT1a, which is generally expressed in all cells, the MFI was compared between the study groups. Figure 14 C shows an applicable CPT1a isotype control tested in order to demonstrate the CPT1a antibody specificity. Here, $CD4^{+}$ effector memory T cells are again exemplarily shown. The CPT1a AlexaFluor®488 antibody (clone 7E10G10, Abcam) binds to the C-terminal region (aa 489-773) of CPT1a at the outer mitochondrial membrane [127].

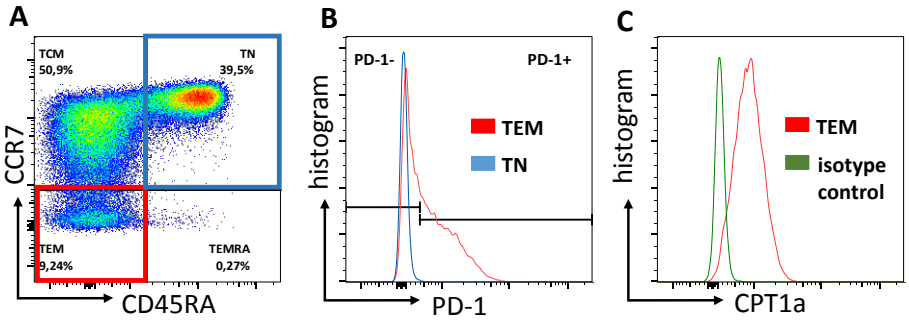

Figure 14: Expression of PD-1 and CPT1a in CD4$^+$ T cell subpopulations. Flow cytometry analyses of cryopreserved PBMCs from MS patients and HC participants. Cells were gated on single cell live lymphocytes as shown in Figure 9: lymphocytes were identified based on size using forward scatter (FSC) and side scatter (SSC) properties. Doublets were excluded based on FSC-H (height) and FSC-A (area) followed by dead cell exclusion. T cells and non-T cells were discriminated based on CD3 surface expression. Further determination of T cells was distinguished by CD4 and CD8 surface expression. CD4$^+$ memory T cell populations are shown here. **(A)** memory T cell subsets were discriminated based on CCR7 and CD45RA surface expression: TCM: CCR7$^+$ CD45RA$^-$, naïve: CCR7$^+$ CD45RA$^+$, TEM: CCR7$^-$ CD45RA$^-$, TEMRA: CCR7$^-$ CD45RA$^+$. **(B)** MFI in CD4$^+$ naïve T cells (blue) as PD-1 negative control applied to all MS and HC T cell subsets analyzed and a representative PD-1 positive staining: CD4$^+$ TEM cells (red). **(C)** MFI of CD4$^+$ TEM CPT1a (red) and its isotype control (green). TEM: effector memory T cells, TN: naïve T cell, TCM: central memory T cell, TEMRA: terminally differentiated effector memory cells re- expressing CD45RA, CCR7: CC chemokine receptor 7.

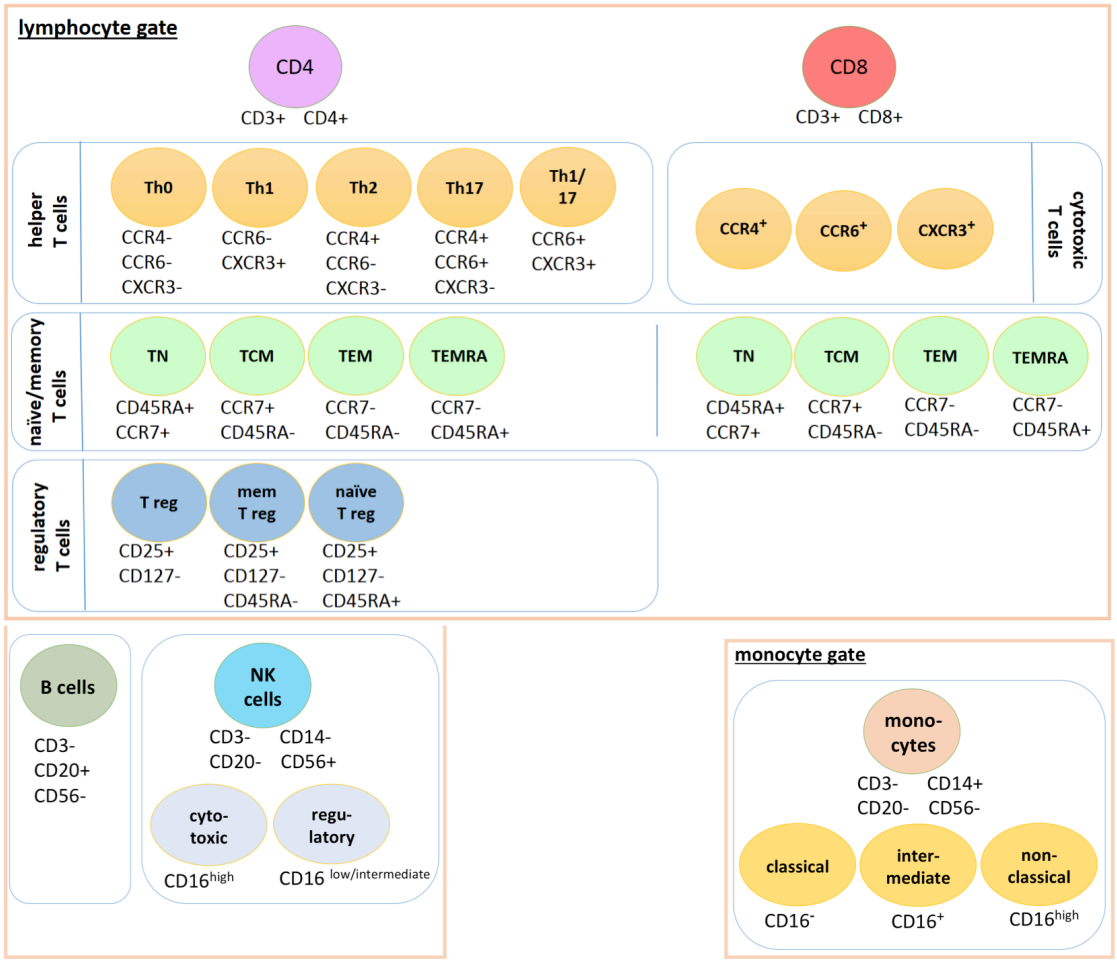

Figure 15: Overview of PBMC phenotyping based on flow cytometry gating in MS patients and HC participants. The lymphocyte gate (top box) and monocytes gate (bottom right box) is set on whole PBMCs differentiated by cell size and width (FSC/SSC), live dead cells, single cells and the respective surface receptors to determine cell phenotypes. The figure illustrates the surface receptors used to differentiate cell populations. CCR: CC chemokine receptor, CXCR3: CXC chemokine receptor 3, CM: central memory, EM: effector memory, TEMRA: terminally differentiated effector memory cells re- expressing CD45RA, NK cell: natural killer cell, cyt NK cell: cytotoxic NK cell, reg NK cell: regulatory NK cell.

2.3.4 Gene Expression Analyses

2.3.4.1 Preparation and Conservation of RNA

In order to obtain RNA samples from $CD4^+$ and $CD8^+$ T cells as well as non-$CD4^+$/$CD8^+$ T cells from MS patients and healthy control participants, aliquots of these three cell fractions were taken after the two hour resting period following the magnetic cell sorting (see 2.3.1.3 and 2.3.1.4). The cells were centrifuged at 300xg for 5 minutes and the supernatant was taken off. 80µL of RNAlater® (Life Technologies) was added to the cell

pellets and resuspended. The cells in RNAlater® solution were stored at 4°C for 1-3 days and frozen at -80°C until analysis.

2.3.4.2 RNA Isolation and cDNA Synthesis

RNA isolation (Qiagen RNeasy® Plus Mini Kit) and cDNA synthesis (RevertAid H Minus First Strand cDNA Synthesis Kit, ThermoFisher Scientific) were performed following the manufacturer's instructions. RNA measurements were performed on a NanoDrop™2000c Spectralphotometer (ThermoFisher, Life Technologies). For each cell fraction from an MS patient and matched healthy control participant, the same quantity of RNA was transcribed into cDNA. cDNA synthesis was performed on a GeneAmp® PCR Systems 9700 (Applied Biosystems, ThermoFisher, Life Technologies). RNA isolation and cDNA synthesis were performed on the same day. The cDNA was stored at -80°C until qRT-PCR analysis.

2.3.4.3 qRT-PCR Analyses

Gene expression analyses from matched MS patients and HC participants were performed on the same qRT-PCR plate to ensure data comparability. For all MS patients and HC participants, $CD4^+$ T cells, $CD8^+$ T cells and non-$CD4^+$/$CD8^+$ T cells were analyzed. TaqMan Gene expression probes and Master Mix (both LifeTechnologies) were used for qRT-PCR analysis on a Step One Plus Real Time PCR System (Applied Biosystems, ThermoFisher, Life Technologies). A full list of all reagents is provided in Table 9 and Table 10.

Per one reaction, the following compounds were used: 10 µL TaqMan Master Mix, 8 µL DEPC water (Life Technologies), 1 µL Taq Man probe and 1 µL cDNA. The samples were analyzed in triplicates with two housekeeping genes (*TATA Box Binding Protein and Importin 8*) analyzed for reference. Following 2 minutes at 50 °C and 10 minutes at 95°C, 40 cycles were performed as follows: 15 seconds at 95°C, 1 minute at 60°C. Finally, 4°C to stop the reaction. The individual gene transcript levels were assessed relative to the housekeeping genes following ΔΔCT values. Differences of more than 0.5 in ΔΔCT values within the triplicates were excluded.

2.3.5 ELISA Analyses of Salivary Cortisol Hormone

MS patients and HC participants collected two morning (after waking up) and two evening (at 9 pm) saliva samples on the two days following the study visit. 30 minutes before the samples were taken, the patients and healthy controls were asked not to: exercise, chew gum, drink anything but water, eat and brush their teeth.
Cortisol levels in saliva of MS patients and HC participants were determined using the IBL Cortisol detection ELISA kit (IBL) according the manufacturer's instructions. MS patient and HC participant sample pairs were analyzed on the same plate.

2.3.6 Statistical Analyses

Data was analyzed using paired-sample statistics based on recommendations for a matched case-control study design [128]. Analyses were performed using SPSS version 23 (IBM Inc.) and GraphPad Prism version 7 (GraphPadSoftware Inc.). FACS Diva version 6.1.2 (BD) and FlowJo® version 10.3 (FlowJo Inc.) was used to conduct flow cytometry analyses, Wave Desktop 2.3 (Agilent) was used to conduct Seahorse analyses (software overview in Table 13). To compare groups and test for statistical significance, paired nonparametric Wilcoxon signed rank test was used presenting median values with interquartile ranges, unless otherwise specified. The Wilcoxon test does not require a normal distribution of data. A two-tailed $p \leq 0.05$; $p \leq 0.01$ and $p \leq 0.001$ was considered significant and a p value ≤ 0.10 was considered a trend. To test for bivariate correlation analyses, Spearman's rank correlation test was used. This test also does not require normal distribution of data. The Spearman's rank correlation coefficient r_S indicates the power and direction (positive or negative correlation) of linear statistical association, providing values between -1 and +1. The p-value shows whether r_S significantly differs from 0. Here, a p-value of $p \leq 0.05$ was considered significant and a p value of ≤ 0.10 was considered a trend.

3 Results

Multiple Sclerosis is a neurodegenerative disease affecting the central nervous system as well as the immune system. Immune cells are dependent on energy resources and it has been shown that energy metabolism greatly affects the general immune cell phenotype including the state of activation, senescence, differentiation, memory development or proliferation [129]. Immune cells respond to external environmental factors in their immediate microenvironment and induce energy metabolic shifts from inside the cell. The immune system of patients diagnosed with RRMS is continuously triggered and repeatedly activated. This chronic immune activation may induce shifts in normal immune cell energy metabolism. Therefore, it was the aim of this thesis to analyze immunometabolic dysfunctions and underlying molecular mechanisms in immune cells of RRMS patients.

3.1 MS Patient and Healthy Participant Cohort Characteristics

MS patients and HC participants were recruited as part on the *Depression and Immune System (DENIM)* study (see *2.1* for full information regarding recruitment and Appendix for full Case Report Form). Main characteristics of MS patients and HC participants are displayed in Table 16. Patients and controls were thoroughly matched for sex, age (± 5 years), BMI (± 2 kg/m^2) and current smoking status. Due to careful matching of patients and controls, there are no differences in sex, age (p=0.43), BMI (p=0.45) or smoking status. The cohort consists primarily of female (77.4%) non-smokers (96.8%).

Psychiatric and cardiovascular diseases have been associated with a general increase in inflammation the body and were therefore excluded in this study to avert influences on results. Other immunological diseases were also exclusion criteria.

The following questionnaires were used for the assessment of major depressive disorder (MDD) and anxiety disorder: Montgomery-Åsperg Depression Rating Scale (MADRS) [130], Mini International Neuropsychiatric Interview German version 5.0.0 DSM-IV (MINI) [131], Beck's Depression Inventory-II (BDI-II) [132, 217] and Beck's Anxiety Inventory (BAI) [133, 216]. Depression scores of MS patients and HC participants were rated in a clinical interview using the MINI and MADRS. The patients and participants included in the

cohorts did not meet MDD criteria (Table 16). The BDI-II and BAI are self-rated questionnaires. MS patients showed significantly higher BDI-II ($p<0.01$) and BAI ($p=0.02$) scores compared to HC participants. However, in both groups, the scores did not meet depression or anxiety disorder criteria. Cognitive ability testing using the Single Digit Modality Scale (SDMT) [134] did not reveal differences between MS patients and HC participants ($p=0.13$). The Expanded Disability Status Scale (EDSS) [135] testing was performed with MS patients to rate the patient's disability status ranging from 0 to 10. The MS patient cohort had a median EDSS of 2 with results ranging from 0 to 4.5 within the cohort.

Blood pressure measurements revealed normal ranges in both cohorts and levels for C-reactive protein (CRP) did not indicate inflammation (Table 16).

In conclusion, MS patients and HC participants did not show any concomitant psychiatric disorders or indicators for pro-inflammation induced by concomitant cardiovascular diseases.

Table 16: MS patients' and healthy control participants' cohort characteristics.

	RRMS (n=31)	**HC (n=31)**	**P***
Age, years	38 (34-48)	40 (30-49)	0.43
BMI, kg/m^2	23.3 (21.3-25.6)	23.7 (22.4-25.8)	0.45
Blood pressure systolic, mmHg	120 (112-131)	123 (106-126)	0.25
Blood pressure diastolic, mmHg	78 (67-87)	78 (69-86)	0.91
CRP, mg/L	0.55 (0.4-1.6)	0.9 (0.4-1.5)	0.45
% Females (n)	77.4 (24)	77.4 (24)	0.99
% Current non-smokers (n)	96.8 (30)	96.8 (30)	0.99
Duration of disease, years	6.15 (2.7-10.9)	n/a	n/a
EDSS	2 (0.5-2.5)	n/a	n/a
SDMT	0.5 (0-1)	0 (-0.5-0.5)	0.13
MADRS	2 (1-3)	1 (0-2)	0.12
BDI-II	4 (1-7)	0 (0-2)	0.01
BAI	3 (1-9)	2 (0-5)	0.02

31 MS patients and 31 matched HC participants were analyzed. CRP was analyzed for n=30 pairs. 24 MS patients added information for MS disease duration. 30 MS patients completed the SDMT. Relapsing Remitting Multiple Sclerosis, HC: Healthy Control, BMI: Body Mass Index, CRP: C-reactive protein, EDSS: Expanded Disability Status Scale, MADRS: Montgomery-Åsperg Depression Rating Scale, BDI-II: Beck's Depression Inventory-II, BAI: Beck's Anxiety Inventory. n/a: not applicable, SDMT: Single Digit Modalities Scale. *Wilcoxon signed-rank test. medians with interquartile ranges (in brackets) are displayed.

As described in 2.1, all MS patients recruited for the study were in remission and on stable immunomodulatory therapy (all inclusion criteria listed in Table 4). The aim was to omit any effects related to non-stable and non-immunomodulatory medication as well as comorbidities in laboratory results. Table 17 provides an overview of all immunomodulatory medications and the corresponding commercial names taken by the MS patients (n=31). Four MS patients did not take immunomodulatory medication.

Table 17: Immunomodulatory medications taken by MS patients.

medication	**commercial name (number of patients)**
Interferon beta 1a	Avonex (2), Rebif 22 (2), Rebif 44 (1)
Interferon beta 1b	Extavir (2)
Teriflunomide	Aubagio (4)
Glatiramer Acetate	Copaxone 20 mg (2), Copaxone 40 mg (4)
Fingolimod	Gilenya (2)
Alemtuzumab	Lemtrada (1)
Dimethyl fumarate	Tecfidera (7)
No medication	(4)

Five patients on additional medications were included due to the year-long stable medication. Amongst them, four patients taking L-Tyroxin or Thyronajod (thyroiditis medication) for more than three years and one patient taking Simvastatin (blood pressure medication) for ten years.

Patients and HC participants were asked during the clinical assessment whether family members are diagnosed with MS. Three female MS patients reported: two cousins (maternal and paternal sides), great-grandmother paternal side and twin sister. One male patient reported that his mother was diagnosed with MS. One female HC participant reported that her sister was diagnosed with MS.

In conclusion, overall, the MS patient cohort was free of immunological, cardiovascular or psychiatric diseases and medications other than the ones taken for MS treatment (with the exception of stable thyroid and blood pressure medication, as stated). Therefore, biological effects measured in laboratory analyses were controlled for as best as possible.

Differential blood analyses showed decreased absolute numbers of lymphocytes in the MS patient cohort ($p=0.01$). No difference in monocytes was measured (Figure 16).

Basophils were decreased in the MS patient cohort (p≤0.01, Figure 35 supplements). Group differences in other cell populations were not observed (see Table 19 in supplements for a complete list of all populations analyzed).

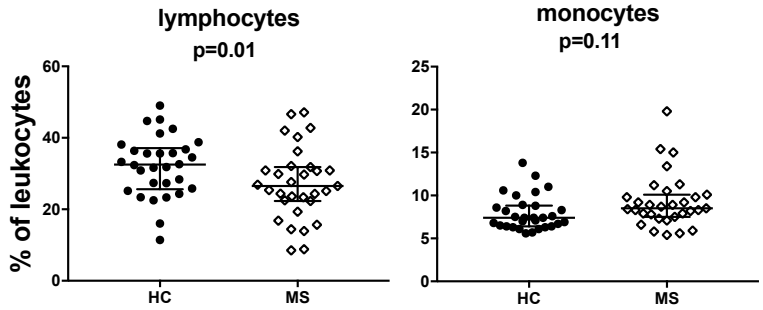

Figure 16: ***Within group comparisons of differential blood parameters in MS patients and HC participants.*** Whole blood was analyzed in a certified routine laboratory: Labor Berlin, Berlin, Germany. Blood was drawn after a 12 hour fasting period and analyzed on the same day. lymphocytes: n=30 pairs. monocytes: n=30 pairs. Wilcoxon signed-rank test with medians with interquartile ranges are displayed. HC: healthy control, MS: multiple sclerosis.

A trend towards decreased high density lipoprotein (HDL) levels in MS patients was observed (p=0.08). Low density lipoprotein (LDL) levels as well as CRP levels did not vary between groups (Figure 17).

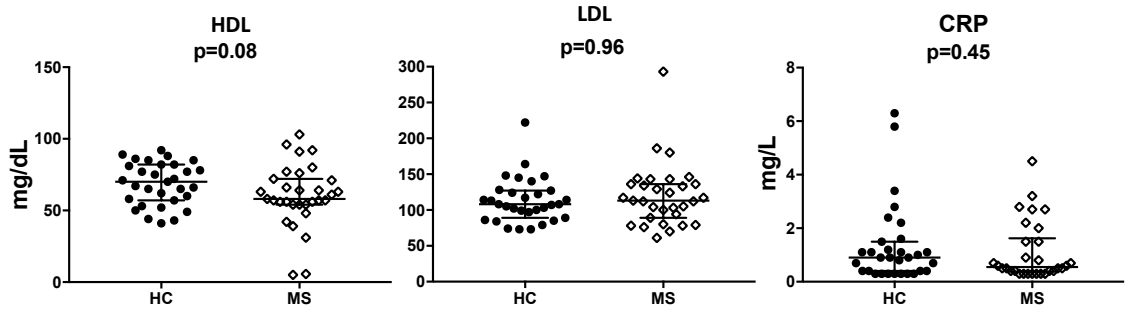

Figure 17: ***Within group comparisons of differential blood parameters HDL, LDL and CRP in MS patients and HC participants.*** Whole blood was analyzed in a certified routine laboratory: Labor Berlin, Berlin, Germany. Blood was drawn after a 12 hour fasting period and analyzed on the same day. HDL: n=31 pairs. LDL: n=31 pairs. CRP: n=30 pairs. Wilcoxon signed-rank test, medians with interquartile ranges are displayed. HDL: high density lipoprotein, LDL: low density lipoprotein, CRP: C-reactive Protein, HC: healthy control, MS: multiple sclerosis.

3.2 In vitro Analysis of Immune Cell Energy Metabolism using the Seahorse XF^{e}96 Analyzer

To analyze and compare immunometabolic profiles in specific PBMC subpopulations, their mitochondrial and glycolytic respiration were assessed. Purified and unstimulated $CD4^+$ T cells, $CD8^+$ T cells and non-$CD4^+$/$CD8^+$ T cells (defined as the cell fraction after $CD4^+$ T cell and $CD8^+$ T cell purification) from MS patients and HC participants were analyzed in real-time using the Seahorse XF^e 96 Analyzer (Agilent, Waldbronn).

3.2.1 Metabolic Assay Verification

To ensure metabolic assay validity and test data compared to available literature on human immune cell energy metabolism, within group comparisons of the real-time energy states of immune cells from the healthy participant cohort were performed.

Figure 18 shows the kinetic profile of $CD4^+$ T cells, $CD8^+$ T cells and non-$CD4^+$/$CD8^+$ T cells from HC participants. Differences in oxygen consumption rate (OCR) are displayed, focusing on basal respiration (BR) and maximal respiration (MR) as exemplary measures. In union with literature on immune cell metabolism, there were no significant differences in BR between resting unstimulated $CD4^+$ T cells and $CD8^+$ T cells (n=24 pairs, p=0.97) as well as $CD8^+$ T cells and non-$CD4^+$/$CD8^+$ T cells (n=23 pairs, p=0.13). $CD4^+$ T cells showed significantly lower basal OCR values compared to non-$CD4^+$/$CD8^+$ T cells (n=24 pairs, $p \leq 0.001$). Analyzing MR, there was a trend between $CD4^+$ T cells and non-$CD4^+$/$CD8^+$ T cells (n=24 pairs, p=0.06) and $CD8^+$ T cells and non-$CD4^+$/$CD8^+$ T cells (n=23 pairs, p=0.07). There was no significant difference in maximal OCR between $CD4^+$ T cells and $CD8^+$ T cells (n=24 pairs, p=0.49).

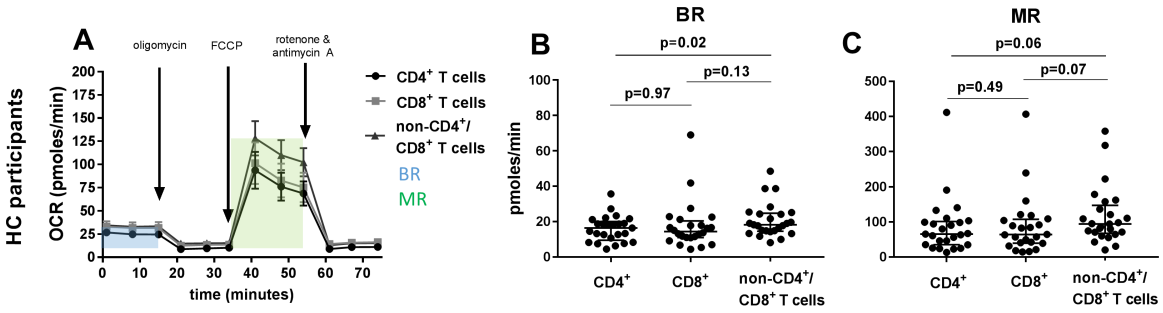

Figure 18: Within group comparisons in the HC participant cohort showed adapted metabolic profiles in CD4$^+$ T cells, CD8$^+$ T cells and non-CD4$^+$/CD8$^+$ T cells. The data was generated in real-time with unstimulated cells under basal conditions, in response to oligomycin, FCCP and Rotenone and Antimycin A using a Seahorse XF^e 96 Analyzer (Agilent). **(A)** kinetic profile of OCR values of CD4$^+$ T cells, CD8$^+$ T cells and non-CD4$^+$/CD8$^+$ T cells. BR and MR are exemplarily colored for non-CD4$^+$/CD8$^+$ T cells. BR measured at third time point before oligomycin injection, MR measured after FCCP injection. **(B)** BR: CD4$^+$ T cells:CD8$^+$ T cells (n=24 pairs), CD4$^+$ T cells:non-CD4+/CD8+ T cells (n=23 pairs), CD8$^+$ T cells:non-CD4$^+$/CD8$^+$ T cells (n=23 pairs). **(C)** MR: CD4$^+$ T cells:CD8$^+$ T cells (n=24 pairs), CD4$^+$ T cells:non-CD4$^+$/CD8$^+$ T cells (n=24 pairs), CD8$^+$ T cells:non-CD4$^+$/CD8$^+$ T cells (n=23 pairs). (A) Mean and SEM calculated per condition for each time point. (B, C) Wilcoxon signed-rank test, medians with interquartile ranges are displayed for BR and MR. OCR: oxygen consumption rate, BR: basal respiration, MR: maximal respiration, FCCP: Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone, HC: healthy control, MS: multiple sclerosis.

In summary, using the Seahorse XF^e 96 Analyzer, current literature data could be confirmed and provided valid results for measuring immunometabolic states of unstimulated human CD4$^+$ T cells, CD8$^+$ T cells and non-CD4$^+$/CD8$^+$ T cells from healthy individuals.

Subsequently, between group analyses of the MS patient and HC participant cohort were performed assessing alterations in immunometabolism in MS disease.

3.2.2 Mitochondrial Energy Metabolism Profile of Purified CD4$^+$ T cells

CD4$^+$ T cells have been shown to play a major role in MS pathogenesis and area main target in current treatment. Therefore, it was of great interest to analyze mitochondrial respiration in this cell population and generate an immunometabolic profile in order to further investigate the role in MS disease.

Following MACS purification, CD4$^+$ T cells from MS patients and HC participants were analyzed in the Seahorse XF^e 96 Analyzer. CD4$^+$ T cells from MS patients showed significantly lower BR ($p \leq 0.001$), MR ($p \leq 0.01$), spare respiratory capacity (SRC) ($p \leq 0.01$) and ATP production ($p \leq 0.01$) compared to HC participants (n=21 to 23 pairs). Non-

mitochondrial respiration (NMR) (p=0.22) and proton leakage (PL) (p=0.13) did not show significant differences between both groups (Figure 19).

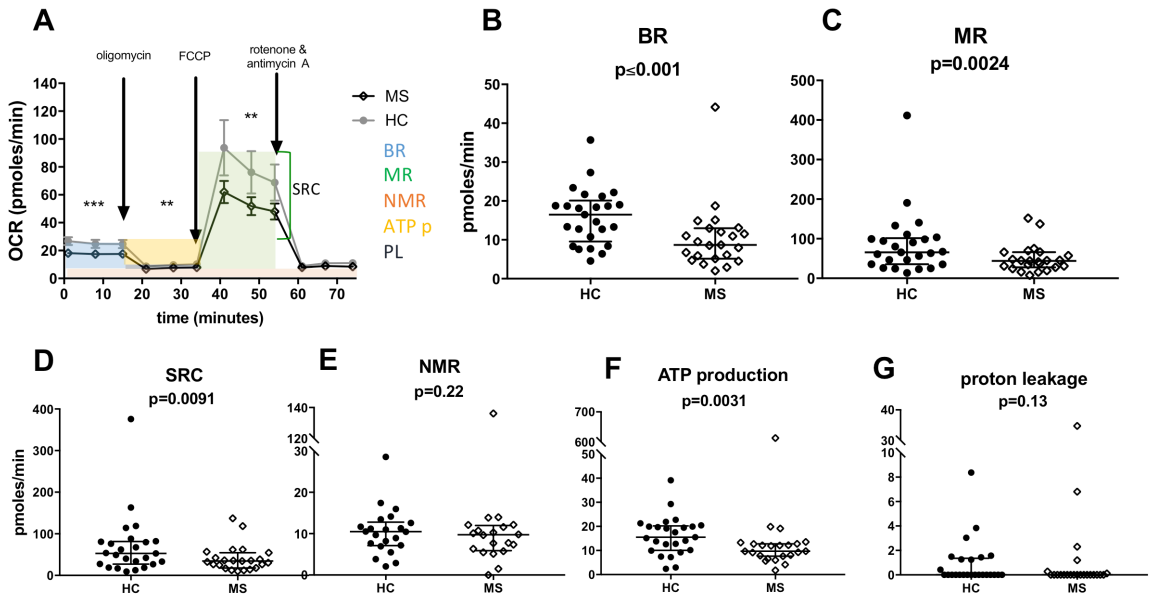

Figure 19: Impaired metabolic profile of CD4$^+$ T cells in MS patients compared to HC participants. The data was generated in real-time under basal conditions, in response to oligomycin, FCCP and Rotenone and Antimycin A using a Seahorse XF^e 96 Analyzer (Agilent). **(A)** Kinetic profile of OCR values of CD4$^+$ T cells. Colored displays are exemplarily shown for HC. **(B)** BR n=23 pairs. **(C)** MR n=22 pairs. **(D)** SRC n=23 pairs. **(E)** NMR n=20 pairs. **(F)** ATP production n=23 pairs. **(G)** PL n=23 pairs, p=0.13. (A) Mean and SEM calculated per group for each time point. (B-G) Wilcoxon signed-rank test, medians with interquartile ranges are displayed. OCR: oxygen consumption rate, BR: basal respiration, MR: maximal respiration, SRC: spare respiratory capacity, NMR: non-mitochondrial respiration, ATP: Adenosine triphosphate, PL: proton leakage, FCCP: Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone, HC: healthy control, MS: multiple sclerosis.

3.2.3 Mitochondrial Energy Metabolism Profile of Purified CD8$^+$ T cells

Complementary to CD4$^+$ T cells, CD8$^+$ T cells have been found to be modulated in MS pathology and are targeted in current MS medication. The aim was to generate a mitochondrial respiration profile of CD8$^+$ T cells from MS patients providing greater insights into autoimmune mechanisms in MS disease and the contributions of energy metabolism of both T cell subpopulations.

CD8$^+$ T cells were purified and prepared for Seahorse XF^e 96 Analyzer measurements. CD8$^+$ T cells from MS patients did not show significant differences in BR, MR, SRC, ATP

production, NMR and PL (all p≥0.26) compared to matched HC participants (n=21 to 23 pairs) (Figure 20).

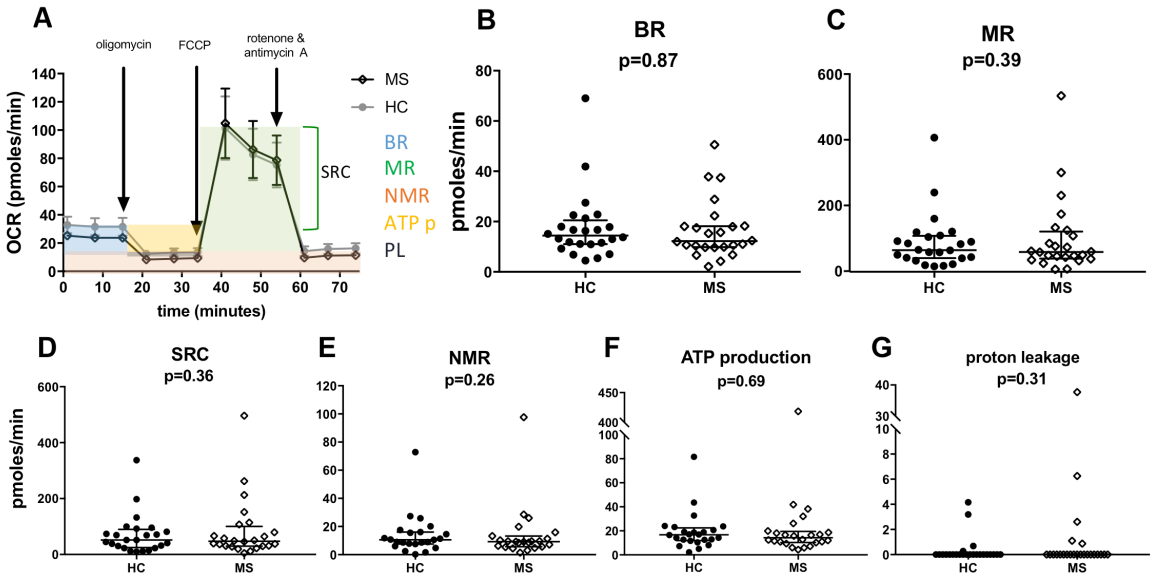

Figure 20: Metabolic profile of mitochondrial respiratory capacity dos not differ in CD8$^+$ T cells from MS patients compared to HC participants. The data was generated in real-time under basal conditions, in response to oligomycin, FCCP and Rotenone and Antimycin A using a Seahorse XF^e 96 Analyzer (Agilent). **(A)** Kinetic profile of OCR values of CD8$^+$ T cells. Colored displays are exemplarily shown for HC. **(B)** BR n=23 pairs. **(C)** MR n=23 pairs. **(D)** SRC n=23 pairs. **(E)** NMR n=21 pairs. **(F)** ATP production n=23 pairs. **(G)** PL n=23 pairs. (A) Mean and SEM calculated per group for each time point. (B-G) Wilcoxon signed-rank test, medians with interquartile ranges are displayed. OCR: oxygen consumption rate, BR: basal respiration, MR: maximal respiration, SRC: spare respiratory capacity, NMR: non-mitochondrial respiration, ATP: Adenosine triphosphate, PL: proton leakage, FCCP: Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone, HC: healthy control, MS: multiple sclerosis.

3.2.4 Mitochondrial Energy Metabolism Profile of non-CD4$^+$/CD8$^+$ T cells

In addition to CD4$^+$ and CD8$^+$ T cells, other immune cells including B cells and NK cells have been shown to contribute to MS disease relapse and progression. Therefore, it was the aim to analyze mitochondrial respiration in the non-CD4$^+$/CD8$^+$ T cell fraction from MS patients as well as HC participants and generate an immunometabolic profile that can be compared to the ones from the CD4$^+$ and CD8$^+$ T cell subpopulations. The non-CD4$^+$/CD8$^+$ T cell fraction mainly constitutes monocytes, B cells as well as NK cells and the analyses

provide first insights into the energy profile of these blood cell populations in MS patients compared to HC participants.

Non-CD4$^+$/CD8$^+$ T cells from MS patients did not show significant differences in BR, MR, SRC, ATP production and NMR (all p≥0.10) compared to matched HC participants (n=21 to 24 pairs) (Figure 21). Proton leakage showed a trend towards an increase in MS patient's non-CD4$^+$/CD8$^+$ T cells (p=0.10).

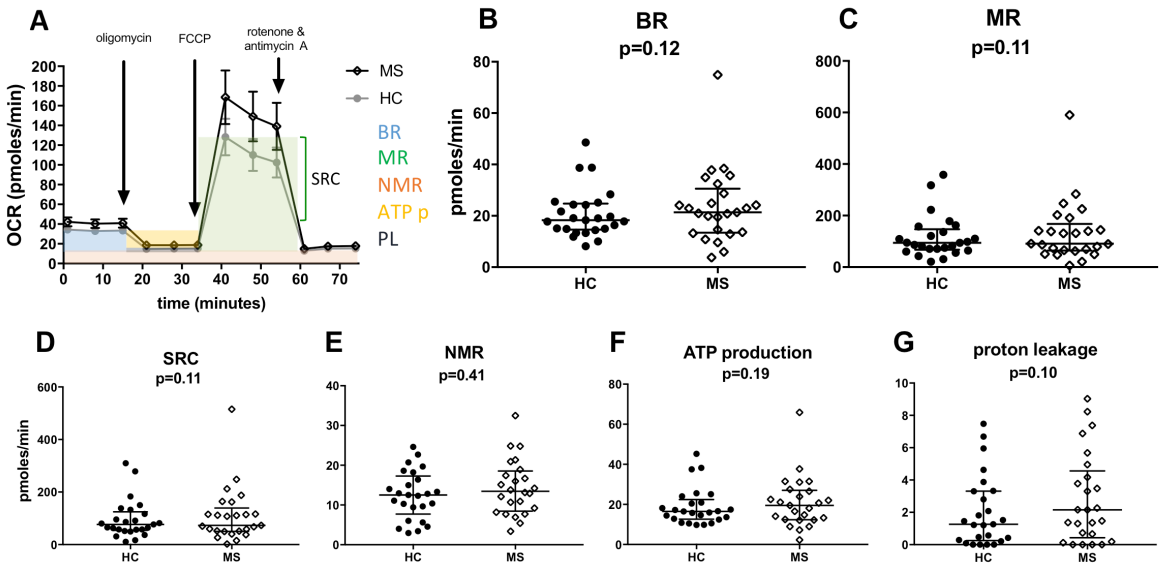

Figure 21: Metabolic profile of mitochondrial respiratory capacity does not differ in non-CD4$^+$/CD8$^+$ T cells from MS patients compared to HC participants. The data was generated in real-time under basal conditions, in response to oligomycin, FCCP and Rotenone and Antimycin A using a Seahorse XF^e 96 Analyzer (Agilent). **(A)** Kinetic profile of OCR values of non-CD4$^+$/CD8$^+$ T cells. Colored displays are exemplarily shown for HC. **(B)** BR n=24 pairs. **(C)** MR n=24 pairs. **(D)** SRC n=24 pairs. **(E)** NMR n=23 pairs. **(F)** ATP production n=24 pairs. **(G)** PL n=24 pairs. (A) Mean and SEM calculated per group for each time point. (B-G) Wilcoxon signed-rank test, medians with interquartile ranges are displayed. OCR: oxygen consumption rate, BR: basal respiration, MR: maximal respiration, SRC: spare respiratory capacity, NMR: non-mitochondrial respiration, ATP: Adenosine triphosphate, PL: proton leakage, FCCP: Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone, HC: healthy control, MS: multiple sclerosis.

In conclusion, the results allude to an overall impaired mitochondrial immunometabolic profile in CD4$^+$ T cells in the MS patient cohort. This was not observed in CD8$^+$ T cells and non-CD4$^+$/CD8$^+$ T cells.

3.2.5 Glycolytic Activity of Purified Immune Cells

In addition to examining mitochondrial respiration, between group analyses of glycolytic activity in $CD4^+$ T cells, $CD8^+$ T cells and non-$CD4^+$/$CD8^+$ T cells was performed for the MS patient and HC participant cohorts. Mitochondrial energy metabolism data of the three cell fractions was complemented with data on glycolytic immunometabolism, thereby testing for compensatory energy pathways and cellular activation status.

Figure 22 summarizes the kinetic profiles as well as basal (measurement three on time axis) and maximal Extracellular Acidification Rate (ECAR) (highest value after oligomycin injection on time axis) for all three cell populations. For comparability purposes with previously shown OCR graphs, all time points are displayed in the kinetic profile. However, time points following FCCP injection were excluded from the analysis of glycolytic activity, because the data compromise additional metabolic pathway activities including glutaminolysis and the pentose phosphate pathway. Activities of these pathways also affect changes in pH in the medium measured by the Seahorse analyzer.
$CD4^+$ T cells from MS patients showed decreased basal ECAR compared to HC participants ($n=24$ pairs, $p\leq0.001$). There was no significant difference in baseline ECAR in $CD8^+$ T cells ($n=23$ pairs, $p=0.32$). Non-$CD4^+$/$CD8^+$ T cells showed a trend toward increased glycolytic activity in MS patients ($n=25$ pairs, $p=0.06$). Maximal ECAR after oligomycin injection was significantly decreased in $CD4^+$ T cells from MS patients ($n=24$ pairs, $p=0.01$), but not in $CD8^+$ T cells ($n=23$ pairs, $p=0.19$) and non-$CD4^+$/$CD8^+$ T cells ($n=24$ pairs, $p=0.37$).

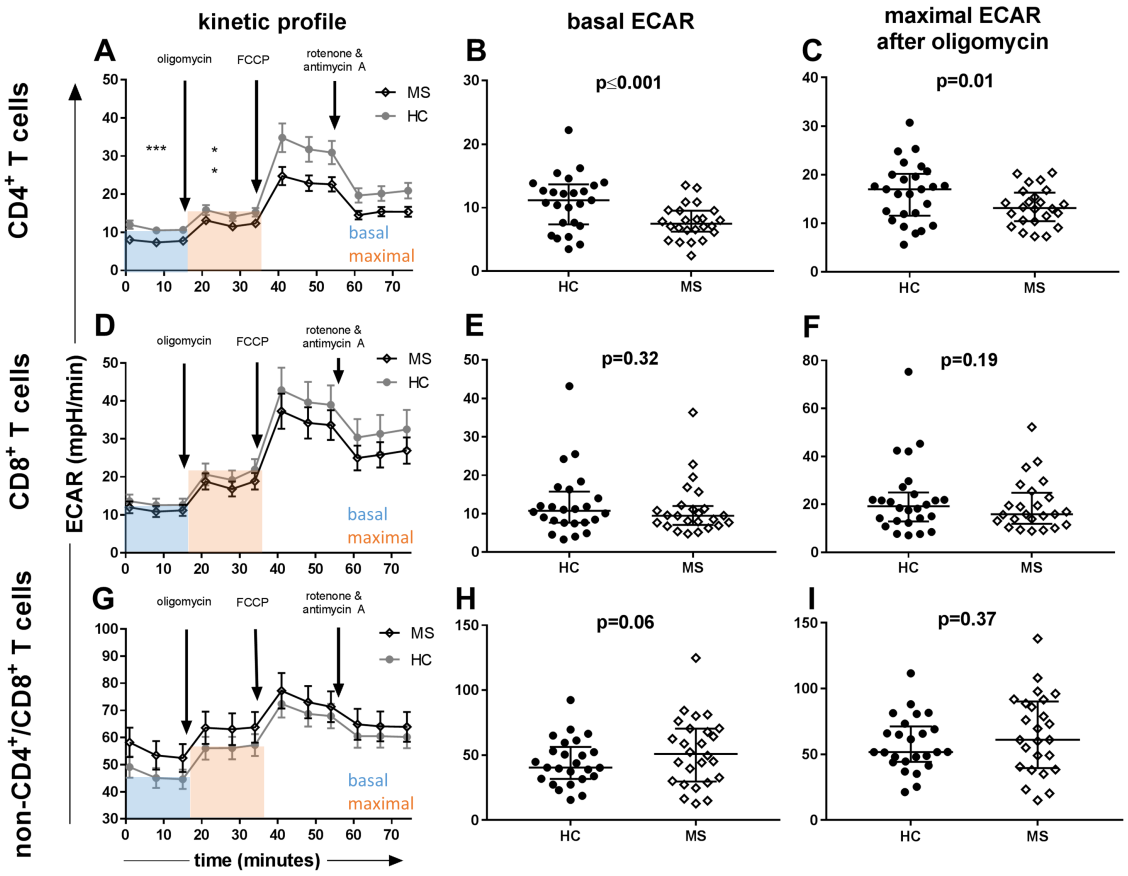

Figure 22: Changes in Extracellular Acidification Rates, between group analyses of MS patient's and HC participant's CD4⁺ T cells, CD8⁺ T cells and non-CD4⁺/CD8⁺ T cells. The data was generated in real-time under basal conditions, in response to oligomycin, FCCP and Rotenone and Antimycin A using a Seahorse XF^{e} 96 Analyzer (Agilent). Kinetic profile of ECAR values of CD4^{+} T cells **(A)**, CD8^{+} T cells **(D)** and non- CD4^{+}/CD8^{+} T cells **(G)**. Basal ECAR in **(B)** CD4^{+} T cells n=24 pairs. **(E)** CD8^{+} T cells n=23 pairs and **(H)** non-CD4^{+}/CD8^{+} T cells n=25 pairs. Maximal ECAR after oligomycin injection in **(C)** CD4^{+} T cells n=24 pairs, **(F)** CD8^{+} T cells n=23 pairs and **(I)** non-CD4^{+}/CD8^{+} T cells n=20 pairs. (A, D, G) Mean with SEM calculated for each time point. (B, C, E, F, H, I) Wilcoxon signed-rank test with medians with interquartile ranges are displayed. ECAR: extracellular Acidification Rate, HC: healthy control, MS: multiple sclerosis.

3.2.6 Metabolic Phenotype of Purified Immune Cells

The previous figures showed mitochondrial and glycolytic activity of purified CD4^{+} and CD8^{+} T cells as well as non-CD4^{+}/CD8^{+} T cells. To summarize and illustrate the changes in OCR and ECAR from basal levels (third value on time axis in previous kinetic graphs) to maximal levels (for OCR after FCCP injection, for ECAR after oligomycin injection on previous kinetic graphs) for each cell population, energy graphs were generated. Figure

23 summarizes the data on cellular immunometabolism in $CD4^+$ T cells, $CD8^+$ T cells and non-$CD4^+$/$CD8^+$ T cells comparing the MS patient and HC participant cohorts.

$CD4^+$ T cells from MS patients not only showed decreased basal and maximal OCR values, but also displayed lower basal and maximal ECAR rates compared to HC participants (Figure 23 A). This alludes to an overall dysfunction in cellular energy metabolism within the $CD4^+$ T cell population in MS patients. $CD8^+$ T cells did not show impairments in OCR and ECAR comparing the MS patient and HC participant cohorts (Figure 23 B). The non-$CD4^+$/$CD8^+$ T cell fraction did not show alterations in OCR values between both groups. In the MS patient cohort, non-$CD4^+$/$CD8^+$ T cells showed a trend toward increased basal ECAR values compared to HC participants, potentially alluding to elevated glycolytic activity in this cell population (Figure 23 C).

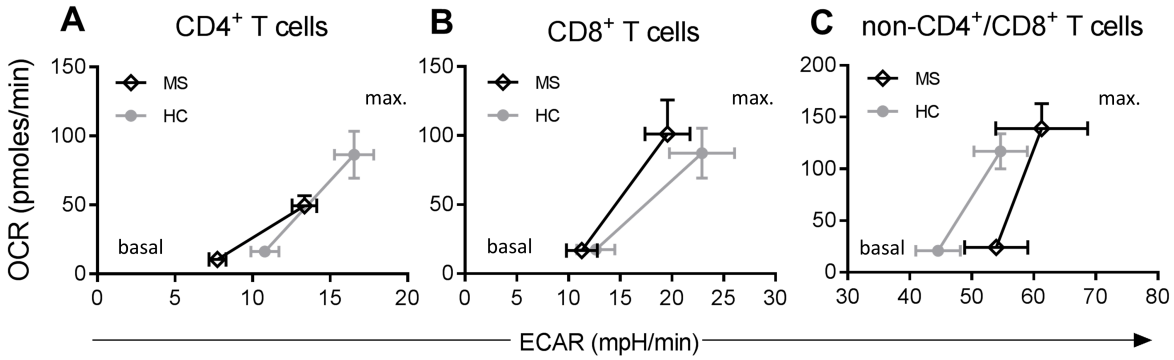

Figure 23: Energy graphs OCR vs. ECAR in $CD4^+$ T cells, $CD8^+$ T cells and non-$CD4^+$/$CD8^+$ T cells in MS patients and HC participants. The data was generated in real-time under basal conditions, in response to oligomycin (maximal ECAR) and FCCP (maximal OCR) using a Seahorse XF^e 96 Analyzer (Agilent). **(A)** $CD4^+$ T cells MS (n=23): basal OCR: 10.5 (1.8), basal ECAR: 7.7 (0.6), max. OCR: 49.5 (7.3), max. ECAR: 13.4 (0.8). $CD4^+$ T cells HC: basal OCR: 16.3 (1.5), basal ECAR: 10.8 (0.9), max. OCR: 86.3 (17.2), max. ECAR: 16.6 (1.3). **(B)** $CD8^+$ T cells MS (n=23): basal OCR: 16.8 (2.5), basal ECAR: 11.3 (1.5), max. OCR: 101.2 (24.7), max. ECAR: 19.6 (2.8). $CD8^+$ T cells HC: basal OCR: 17.5 (2.9), basal ECAR: 12.6 (1.8), max. OCR: 87.2 (18.1), max. ECAR: 22.9 (3.2). **(C)** non-$CD4^+$/$CD8^+$ T cells MS (n=24): basal OCR: 24.3 (2.9), basal ECAR: 54.0 (5.1), max. OCR: 139.1 (24.2), max. ECAR: 61.3 (7.4). non-$CD4^+$/$CD8^+$ T cells HC: basal OCR: 20.9 (2.0), basal ECAR: 44.6 (3.6), max. OCR: 117.0 (17.0), max. ECAR: 54.7 (4.3). Means with SEM (in brackets) calculated. For some points, the error bars would be shorter than the height of the symbol, therefore, in these cases the error bars were excluded. OCR: Oxygen Consumption Rate, ECAR: extracellular Acidification Rate, max.: maximal, HC: healthy control, MS: multiple sclerosis.

In summary, comprehensive immunometabolic profiles of enriched $CD4^+$ and $CD8^+$ T cells as well as non- $CD4^+$/$CD8^+$ T cells were generated for the MS patient and HC participant

cohorts. Two main cellular energy pathways were analyzed, mitochondrial respiration as well as glycolytic respiration, providing central insights into immunometabolism in MS disease.

To further investigate the complexity of immune cell subpopulations and gain insight into specific cell signaling, flow cytometry analyses of MS patient's and HC participant's PBMCs were performed.

3.3 Flow Cytometry Analyses

Metabolic analyses showed impaired energy profiles in immune cells from MS patients. Predominantly, enriched $CD4^+$ T cells from MS patients displayed an overall decrease in mitochondrial and glycolytic activity. To investigate the phenotype of specific cell subpopulations, PBMCs were analyzed by flow cytometry. Complementing the phenotypic characterization of the PBMCs, the analysis of a potential impairment in mitochondrial respiration as well as T cell exhaustion was of great interest. Therefore, the mitochondrial membrane protein CPT1a, essential in fatty acid transfer into mitochondria, and the cellular exhaustion marker PD-1, upregulated in ongoing immune cell activation, were assessed. Phenotypic characterization as well as CPT1a and PD-1 target analyses provide more detailed information about the underlying mechanisms of modulations in immunometabolism.

3.3.1 Phenotypic Characterization of Immune Cell Subpopulations

The phenotype of PBMCs from MS patients and their correspondingly matched HC participants was analyzed pairwise in flow cytometry. Antibody staining panels are listed in Table 12. There were no significant differences in the relative number of $CD4^+$ T cells ($p=0.59$), $CD8^+$ T cells ($p=0.24$), regulatory T cells (T regs) ($p=0.86$) as well as classical ($p=0.68$), intermediate ($p=0.56$) and non-classical ($p=0.54$) monocytes between MS patients and HC participants ($n=29$ pairs) (Figure 24).

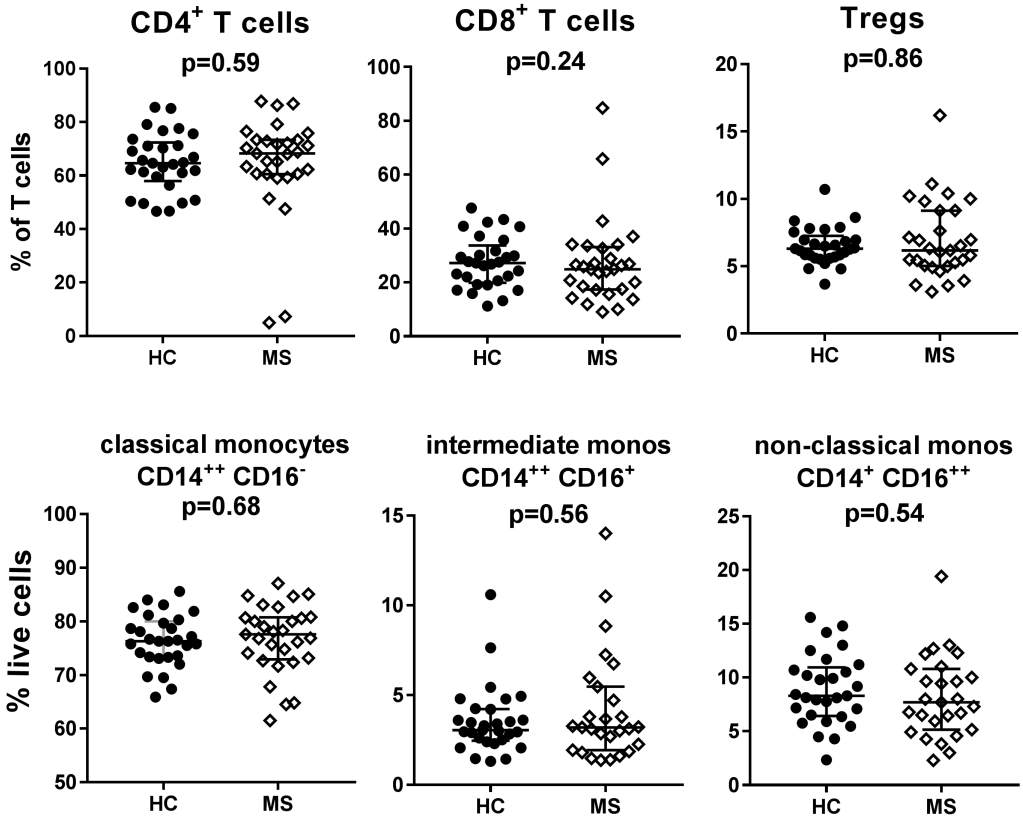

Figure 24: T cell and monocyte subpopulations show no differences in frequency between MS patients and HC participants. Between group analyses of MS patients and HC participants, n=29 pairs. T cells are % of live T cells in $CD3^+$ T cell population, monocytes are % of live monocytes in FSC/SSC monocyte gate. $CD4^+$ T cells p=0.59, $CD8^+$ T cells, Tregs, $CD14^+$ $CD16^-$ classical monocytes, $CD14^+$ $CD16^+$ intermediate monocytes and $CD14^+$ $CD16^{+high}$ non-classical monocytes. Wilcoxon signed-rank test, medians with interquartile ranges are displayed. , HC: healthy control, MS: multiple sclerosis.

$CD4^+$ central memory (CM), effector memory (EM), terminally differentiated effector memory cells re- expressing CD45RA (T_{EMRA}) and naïve T cells (T_N) do not show significant differences in % of $CD4^+$ T cells between MS patients and HC participants (all p≥0.52, n=28 pairs) (appendix Figure 36). $CD8^+$ T_{CM} show a trend towards being decreased within the $CD8^+$ T cell population in MS patients compared to HC participants (p=0.06, n=28 pairs). T_{EM}, T_{EMRA} and T_N $CD8^+$ T cells do not show significant differences in % of $CD8^+$ T cells (all p≥0.32, n=28 pairs) (appendix Figure 36).

Comparing and analyzing frequencies of T cell subtypes based on the expression of chemokine receptors, MS patients show a decreased frequency of $CD8^+$ $CCR6^+$ T cells compared to HC participants (n=29 pairs, p=0.03, appendix Figure 37). Additional chemokine receptor expressing $CD4^+$ helper and $CD8^+$ effector T cell subpopulations did

not show statistically significant differences in frequencies between both cohorts (all p≥0.31, appendix Figure 37).

Analyzing non T cell populations, an increased amount of B cells was detected in MS patients' PBMCs (n=29 pairs, p≤0.01, appendix Figure 38). Furthermore, the frequency of cytotoxic NK cells was significantly decreased (p≤0.001) and regulatory NK cells were significantly increased in MS patients (p≤0.001, appendix Figure 39).

In conclusion, no major differences in frequencies of $CD4^+$ and $CD8^+$ T cell subpopulations or monocyte subsets were detected comparing the MS patient's and HC participant's cohorts. Alterations of B cell and NK cell populations could be shown in MS patients.

3.3.2 CPT1a Expression in Immune Cell Subpopulations

Complementing phenotypic analyses, intracellular CPT1a protein staining was performed in all cell subtypes to further investigate metabolic functioning.

To confirm the specificity of the CPT1a antibody, its isotype control was used to stain PBMCs from an MS patient-HC participant pair. Figure 25 displays exemplary histograms in major T cell subpopulations comparing the CPT1a antibody and isotype control staining. The isotype control stainings did not show detectable background fluorescence, which could allude to non-specific FC-receptor binding or protein interactions. These results are representative for other PBMC subpopulations.

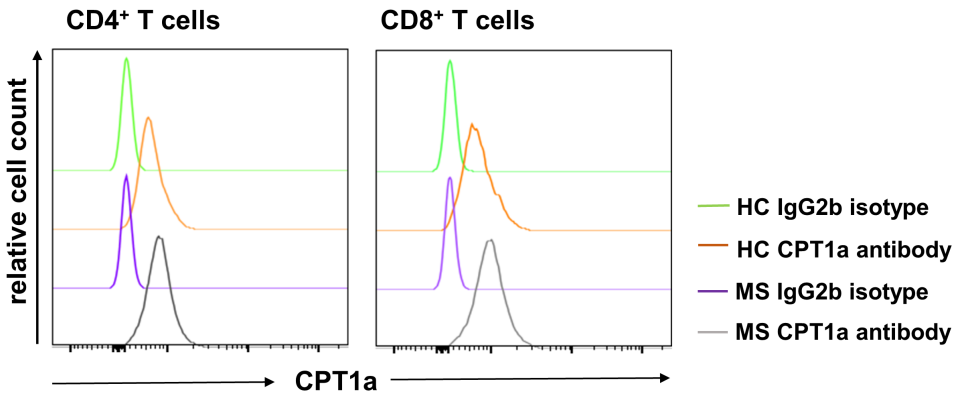

Figure 25: Flow cytometry isotype control staining for CPT1a coupled AlexaFluor®488 antibody in $CD4^+$ and $CD8^+$ T cells. Histogram show stainings of PBMC subpopulations from an MS patient-HC participant pair using the CPT1a antibody (AlexaFluor®488, clone 8F6AE9, Abcam) and its isotype control (mouse IgG2b AlexaFluor®488, clone 7E10G10, Abcam). CPT1a: Carnitine palmitoyltransferase I isoform, HC: healthy control, MS: multiple sclerosis.

Total CD4$^+$ T cells and CD4$^+$ helper T cell populations, Th0, Th1, Th2, Th17 and Th1/17 cells, showed increased CPT1a median fluorescent intensity (MFI) in MS patients compared to HC participants (all p≤0.02, n=29 pairs) (Figure 26 A, C). A representative MS patient-HC participant histogram is shown for each cell population (Figure 26 B, D).

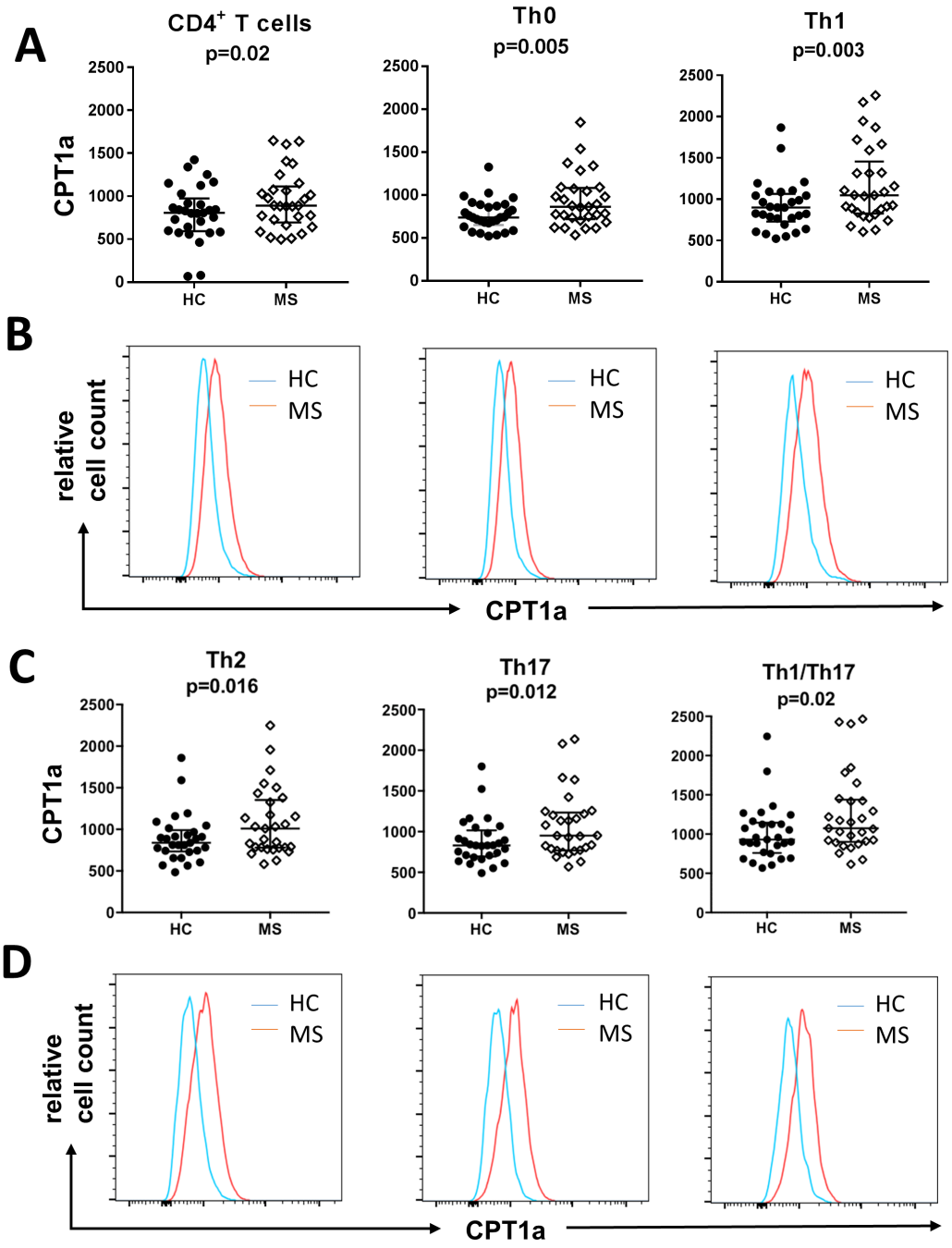

Figure 26: CD4$^+$ T cells and CD4$^+$ helper T cell subpopulations show increased MFI of CPT1a analyzed by flow cytometry. **(A,C)** Between group analyses for MS patients and HC participants are shown, n=29 pairs. **(B, D)** Histograms show CPT1a expression in a representative MS patient-HC participant pair in the corresponding cell subsets. Wilcoxon signed-rank test, medians with interquartile ranges are displayed. CPT1a: Carnitine palmitoyltransferase I isoform, Th: T helper cell, MFI: Median Fluorescent Intensity, HC: healthy control, MS: multiple sclerosis.

There were no differences in CPT1a MFI measured in naïve, EM, CM or TEMRA subpopulations in $CD4^+$ as well as in $CD8^+$ T cells between MS patients and matched HC participants (all p≥0.15, n=28 pairs) (appendix Figure 40).

$CD4^+$ T regs and the subpopulation of naïve T regs showed increased CPT1a MFIs in MS patients (p≤0.023, n=29 pairs). Memory T regs showed a trend toward increased CPT1a MFI in MS patients (p=0.066, n=29 pairs) (Figure 27 A). A representative MS patient-HC participant histogram is shown for each subpopulation (Figure 27 B).

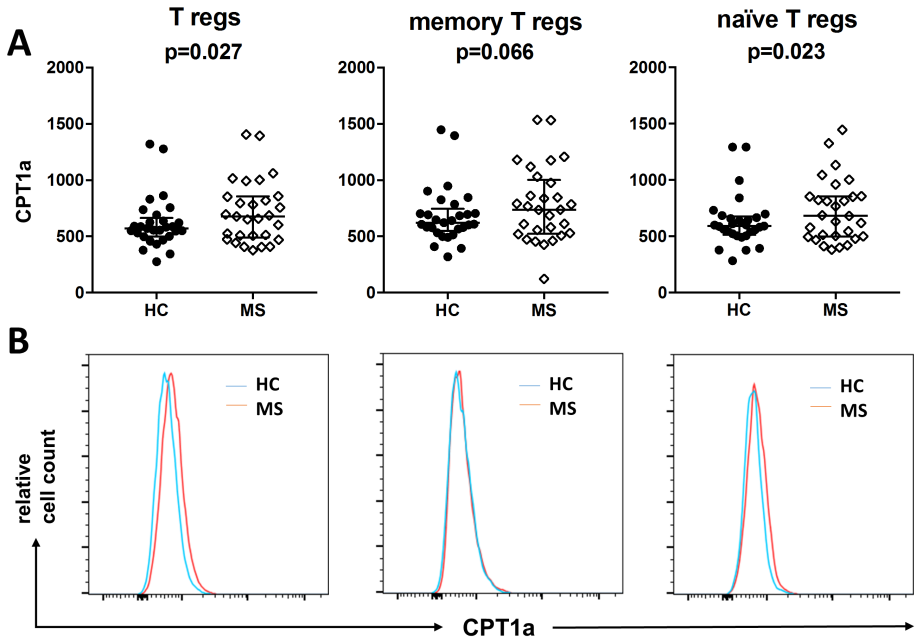

Figure 27: $CD4^+$ regulatory T cell subpopulations show increased CPT1a MFIs as analyzed by flow cytometry. **(A)** Between group analyses for MS patients and HC participants are shown. n=29 pairs. **(B)** Histograms show CPT1a expression in the corresponding cell subsets of a representative MS patient-HC participant pair. Wilcoxon signed-rank test, medians with interquartile ranges are displayed. CPT1a: Carnitine palmitoyltransferase I isoform, T reg: regulatory T cell, MFI: Median Fluorescent Intensity, HC: healthy control, MS: multiple sclerosis.

When analyzing $CD8^+$ T cell subpopulations, it was shown that total $CD8^+$ T cells did not differ in CPT1a MFI (p=0.59, n=29 pairs), while the subsets of $CCR4^+$ T cells, $CCR6^+$ T cells and $CXCR3^+$ T cells showed increased CPT1a MFIs in MS patients (all p≤0.02, n=29 pairs) (Figure 28 A). This concludes that there may be $CD8^+$ T cell sub-populations that did not differ in or displayed decreased CPT1a MFI between the MS patient and HC participant groups. It may most likely be that these cells are non-chemokine receptor expressing cells. Histograms from representative case-control pairs are shown for each cell population (Figure 28 B).

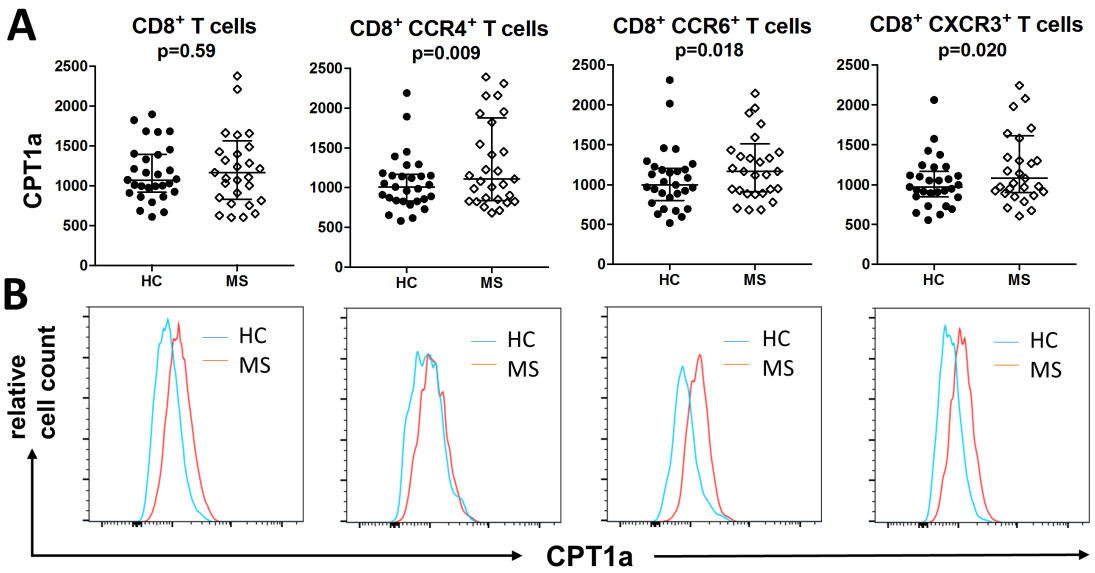

Figure 28: CD8$^+$ T cell subpopulations showed increased MFI for CPT1a in flow cytometry. (A) Between group analyses for MS patients and HC participants are shown. n=29 pairs. CD8$^+$ T cells. CD8$^+$ CCR4$^+$ Tcells. CD8$^+$ CCR6$^+$ T cells. CD8$^+$ CXCR3$^+$ T cells. **(B)** Corresponding histograms show a presentative MS patient-HC participant pair MFI of CPT1a vs. cell count for each cell population. Wilcoxon signed-rank test, medians with interquartile ranges are displayed. CCR: CC chemokine receptor, CXCR3: CXC chemokine receptor 3, MFI: Median Fluorescent Intensity, HC: healthy control, MS: multiple sclerosis.

Furthermore, CPT1a MFI was analyzed in B cells, NK cell subpopulations and monocyte subpopulations. MS patients showed increased CPT1a MFI in cytotoxic NK cells (n=29 pairs, p≤0.05) and a trend of increased CPT1a in regulatory NK cells (n=29 pairs, p=0.057). B cell and monocyte subpopulations did not differ compared to the HC cohort (appendix Figure 41 A).

In conclusion, CD4$^+$ helper T cells, T regs, naïve T regs as well as CD8$^+$ T cell subpopulations and cytotoxic NK cells showed increased levels of CPT1a in the MS patient cohort compared to the HC participant cohort.

These results suggest a dysfunction in mitochondrial energy regulation in MS patient's immune cell subtypes, predominantly in CD4$^+$ T cell subpopulations and therefore add to the results obtained in the Seahorse analyses with a decrease in mitochondrial and glycolytic activity observed in CD4$^+$ T cells.

3.3.3 PD-1 Expression in Immune Cell Subpopulations

To investigate whether PBMCs from MS patients indicate cellular exhaustion phenotypes, cell surface PD-1 (programmed cell death-1) levels were measured. Overall, there were

no significant differences in PD-1 expression measured in any T cell population. Figure 29 shows PD-1 MFIs for $CD4^+$ T cells (p>0.1, n=29 pairs) and $CD8^+$ T cells (p>0.1, n=29 pairs).

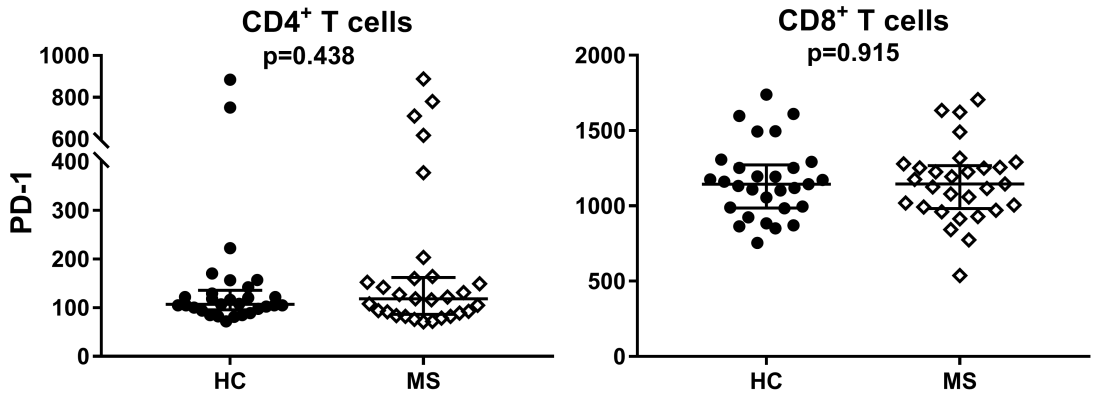

Figure 29: PD-1 expression levels in $CD4^+$ and $CD8^+$ T cells from MS patients and HC participants. Within group analyses of MS patients and HC participants (n=29 pairs). $CD4^+$ T cells and $CD8^+$ T cells. Wilcoxon signed-rank test, medians with interquartile ranges are displayed. HC: healthy control, MS: multiple sclerosis.

PD-1 expression analyses in $CD4^+$ and $CD8^+$ T cell subpopulations (naïve, memory, effector and helper T cells as well as T regs) did not show changed patterns comparing the MS patient and HC participant cohorts.

Taken together, the results indicate that PD-1 expression patterns are not altered in T cell subpopulations from MS patients compared to HC participants and therefore do not indicate a PD-1-mediated cellular exhaustion phenotype.

3.3.4 IL7-Rα and IL2-Rα expression in conventional T cells

IL7-Rα (CD127) and IL2-Rα (CD25) play essential roles in $CD4^+$ T cell development and homeostasis. In this study, the markers were primarily used to discriminate T regs from $CD4^+$ $CD25^-$ $CD127^+$ conventional T cells. In addition to using IL7-Rα and IL2-Rα for phenotyping, the MFI was analyzed in $CD4^+$ $CD25^-$ $CD127^+$ conventional T cells, which provided information about mature $CD4^+$ T cell maintenance, activation, survival and T cell receptor signaling excluding T regs. The results showed a trend toward decreased IL7-Rα and no changes in IL2-Rα expression in $CD4^+$ $CD25^-$ $CD127^+$ conventional T cells of MS patients compared to HC participants (Figure 30). The histogram shows a representative

MS patients and HC participant pair for IL7-Rα (Figure 30 A). IL7-Rα (n=29 pairs, p≤0.1) and IL2-Rα (n=28 pairs, p=0.844) (Figure 30 B and C).

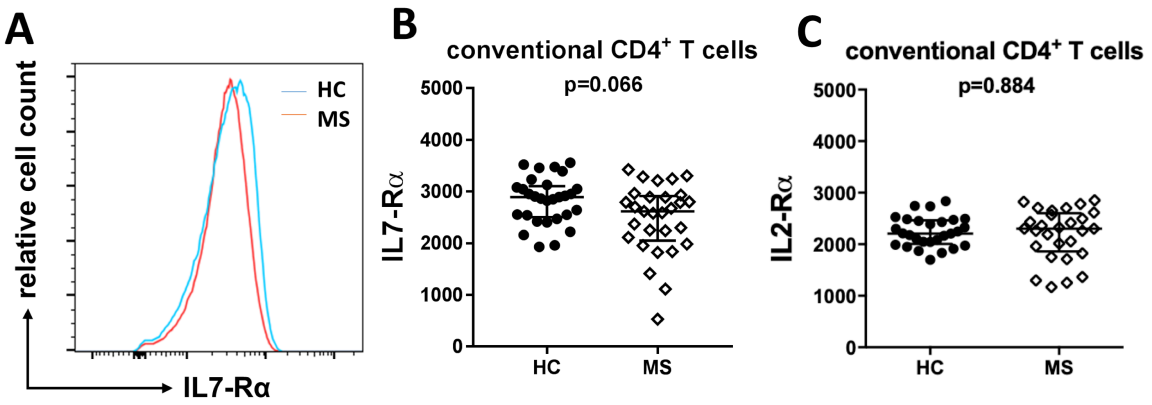

Figure 30: Conventional CD4$^+$ T cells show a trend towards decreased IL7-Rα and no changes in IL2-Rα expression in MS patients compared to HC participants. **(A)** The histogram shows IL7-Rα (CD127) expression in conventional T cells from a representative MS patient-HC participant pair. Between group analyses of MFI in conventional T cells of MS patients and HC participants, IL7-Rα n=29 pairs, p=0.066 **(B)** and IL2-Rα n=28 pairs, p=0.844 **(C)**. Wilcoxon signed-rank test, medians with interquartile ranges are displayed. Conventional T cells are defined as all CD4$^+$ T cells excluding CD25$^+$ CD127$^+$ T regs. T regs: regulatory T cells, conventional T cells: CD4$^+$ CD25$^-$ CD127$^+$ conventional T cells, HC: healthy control, MS: multiple sclerosis.

3.4 mRNA Gene Expression in CD4$^+$ T cells, CD8$^+$ T cells and non-CD4$^+$/CD8$^+$ T cells

Complementing the metabolic analyses with the Seahorse analyzer and the phenotypic, CPT1a and PD-1 protein level analyses by flow cytometry, gene expression was measured in MS patient's and HC participant's CD4$^+$ T cells, CD8$^+$ T cells and non-CD4$^+$/CD8$^+$ T cells (defined as all PBMCs except for CD4$^+$ and CD8$^+$ T cells). In mRNA gene expression analyses, mechanisms like post-translational protein modifications can be omitted and direct gene transcription measured.

Linking pro-inflammatory signaling pathways along with energy metabolism activation pathways were the main interest. Therefore, genes encoding key cellular signaling proteins that integrate metabolism and inflammation were chosen for analysis and mRNA expression levels were measured. The genes of interest were *tumor necrosis factor-α (TNF-α), glucose transporter 1 (GLUT1), Carnitine palmitoyltransferase I isoform (CPT1a), nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NFκB) 1* and *NFκB3.*

Additionally, to further investigate the impact and relevance of stress response systems on immune cells in MS disease and to accompany data from circadian hypothalamic-pituitary-adrenal (HPA) axis activity measured by salivary cortisol, mRNA expression levels of the *glucocorticoid receptor* (*GR*) and the *glucocorticoid-induced leucine zipper* (*GILZ*) were measured in all three cell fractions.

3.4.1 mRNA Analysis of Pro-Inflammatory and Energy Metabolism Genes TNF-α, NFκB1, NFκB3, GLUT1 and CPT1a

Pair-wise within group analyses of *TNF-α, GLUT1, CPT1a, NFκB1* and *NFκB3* mRNA gene expression levels between the MS patient cohort and HC participant cohort were performed. There were no significant differences in mRNA expression levels of the analyzed genes in $CD4^+$ T cells (n=26 pairs, $p>0.1$) and $CD8^+$ T cells (n=26 pairs, $p>0.1$). In non-$CD4^+$/CD8+ T cells, *TNF-α* levels were elevated in MS patients (n=25 pairs, $p\leq0.05$) and there was a trend toward increased CPT1a expression levels in MS patients (n=25 pairs, $p\leq0.1$), while the other genes did not show differing expression patterns (n=23 pairs, all $p>0.1$) (Figure 31).

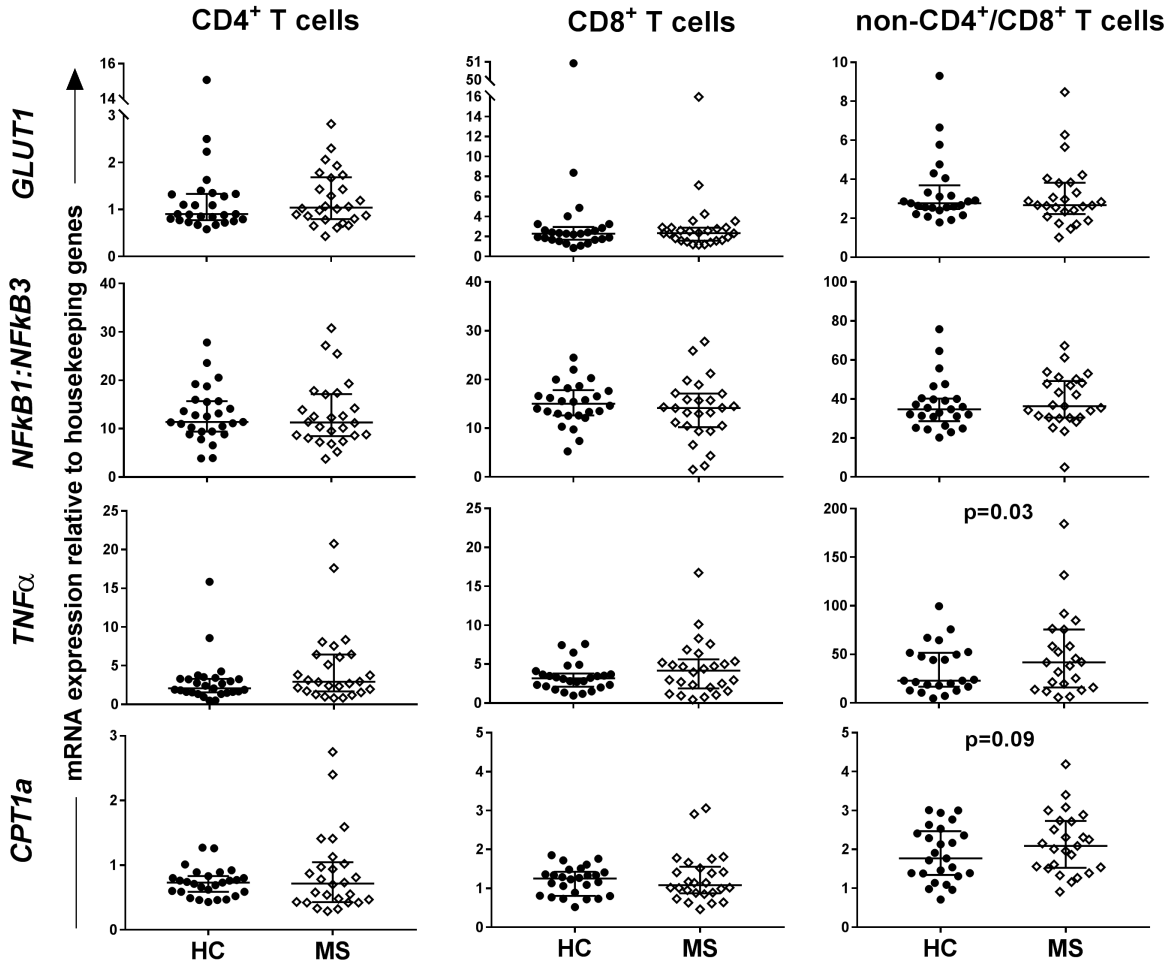

Figure 31: Analysis mRNA gene expression in CD4+ T cells, CD8+ T cells and non-CD4+/CD8+ T cells from MS patients and HC participants. Expression analyses for *GLUT1, NFκB1, NFκB3, TNFα* and *CPT1a* are shown. The ratio between *NFκB1* and *NFκB3* was determined for *NFκB* expression. $CD4^+$ T cells: n=26 pairs, p>0.1 for all genes analyzed. $CD8^+$ T cells: n=26 pairs, p>0.1 for all genes analyzed. non-$CD4^+$/$CD8^+$ T cells: n=25 pairs, p>0.1 if not depicted. Gene transcript levels were assessed relative to housekeeping genes *IPO8* and *TBP* following ΔΔCT. Wilcoxon signed-rank test with medians with interquartile ranges are displayed. *CPT1a: Carnitine palmitoyltransferase I isoform a, TNF-α: Tumor Necrosis Factor-α, NF-κB: nuclear factor 'kappa-light-chain-enhancer' of activated B-cells, GLUT1: Glucose Transporter 1, IPO8: Importin 8, TBP: TATA Box Binding Protein,* HC: healthy control, MS: multiple sclerosis.

3.4.2 mRNA Analysis of Glucocorticoid Receptor (GR) and Glucocorticoid-induced Leucine Zipper (GILZ)

GR and *GILZ* gene expression were analyzed in $CD4^+$ T cells, $CD8^+$ T cells and non-$CD4^+$/$CD8^+$ T cells from MS patients and HC participants. Significant differences were observed in *GILZ* mRNA expression levels in $CD4^+$ T cells with higher gene expression in

MS patients (n=27 pairs, p≤0.05) and in *GR* mRNA expression levels in $CD8^+$ T cells with lower gene expression in MS patients (n=27 pairs, p≤0.01). In non-$CD4^+$/$CD8^+$ T cells, there were no group differences in *GR* or *GILZ* gene expression (Figure 32).

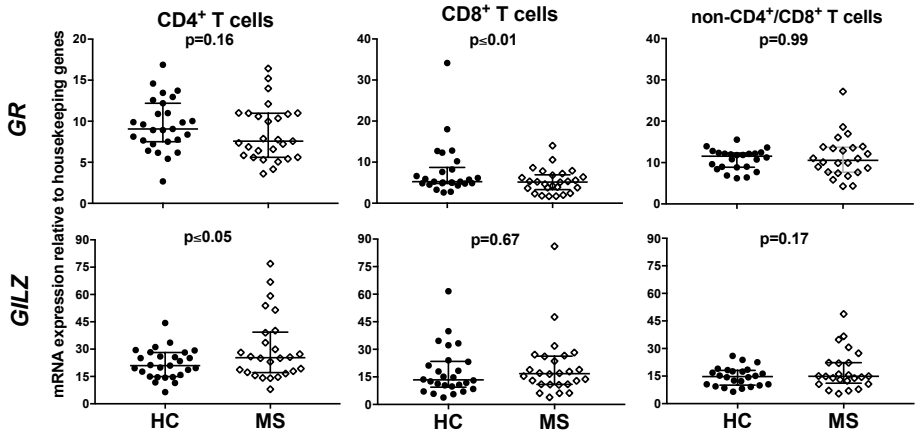

Figure 32: Analysis GR and GILZ mRNA gene expression in $CD4^+$ T cells, $CD8^+$ T cells and non-CD4+/CD8+ T cells from MS patients and HC participants. $CD4^+$ T cells: *GR* n=26 pairs, *GILZ* n=27 pairs. $CD8^+$ T cells: GR and GILZ n=26 pairs. non-$CD4^+$/$CD8^+$ T cells: GR and GILZ n=24 pairs. Gene transcript levels were assessed relative to housekeeping genes *IPO8* and *TBP* following ΔΔCT. Wilcoxon signed-rank test with medians with interquartile ranges are displayed. *GR: Glucocorticoid Receptor*, *GILZ: Glucocorticoid-induced Leucine Zipper, IPO8: Importin 8, TBP: TATA Box Binding Protein*, HC: healthy control, MS: multiple sclerosis.

3.5 Cortisol Levels in Saliva Samples

To analyze circadian hypothalamic-pituitary-adrenal (HPA) axis activity, salivary cortisol levels were measured at four time points on the two consecutive days following the study site visit (morning and evening). 25 MS patient-HC participant pairs were analyzed (Figure 33). There were no significant differences between the two groups at morning or evening measures (p>0.1). A significant difference was measured between morning and evening samples across both groups showing increased cortisol levels after awakening (p<0.001).

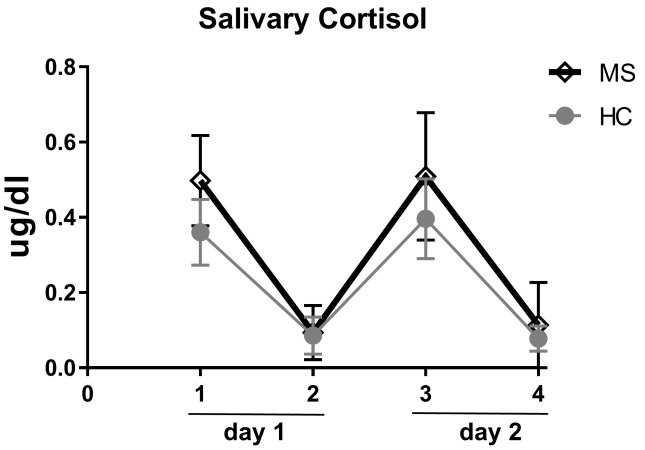

Figure 33: No difference in morning and evening salivary cortisol levels between MS patients and HC participants. On two consecutive days, two morning (after awakening, time point 1 and 3) and two evening (at 9pm, time point 2 and 4) saliva samples were analyzed. n=25 pairs. Day one, morning: p=0.10, evening: p>0.99. Day two, morning: p=0.24, evening: p=0.96. A significant difference in cortisol levels was measured between morning and evening cortisol levels (*p<0.001*) for both groups. 2x4 repeated measures Anova, 95% confidence interval. HC: healthy control, MS: multiple sclerosis.

3.6 Clinical Data Correlation Analyses

Correlation analyses were performed in order to observe associations between case report form (CRF) data and metabolic and flow cytometry results. From CRF data, the MS progression index was chosen for correlation analyses. It is calculated by dividing the Expanded Disease Status Scale (EDSS) score by disease duration (the time between the clinical MS diagnosis and date of the study visit) [136]. The EDSS allows the rating of disease progression on a scale from 0 to 10 and thereby defines the patient's disability status based on the physical functional assessment. The disease duration takes into account for how long the patients have been diagnosed with MS. Therefore, the MS progression index not only provides information about MS duration, but also takes into account the patient's disability status.

To recollect briefly, in this study, the median EDSS score was 2 (with the interquartile range (IQR) of 0.5-2.5) and the median disease duration was 6.15 years (IQR 2.7-10.9 years) (Table 16).

Spearman's rho correlation analyses between MS progression index and flow cytometry CPT1a levels in $CD4^+$ T cells revealed a trend for a negative correlation with lower CPT1a levels in MS patients with higher disability scores (r_S=-0.40, p=0.06) (Figure 34 A).

Correlation analyses of CPT1a in $CD8^+$ T cells and MS progression index revealed a negative correlation (r_S=-0.52, p=0.01) (Figure 34 B). Similar to $CD4^+$ T cells, in $CD8^+$ T cells, CPT1a decreases with increasing progression index.

Correlation analyses between MS progression index and Seahorse XF^e 96 metabolic analyzer basal respiration data were performed for $CD4^+$ and $CD8^+$ T cell subtypes (r_S=0.13, p=0.61 and r_S=-0.30, p=0.92 respectively). These analyses did not reveal correlations (Figure 34 C and D).

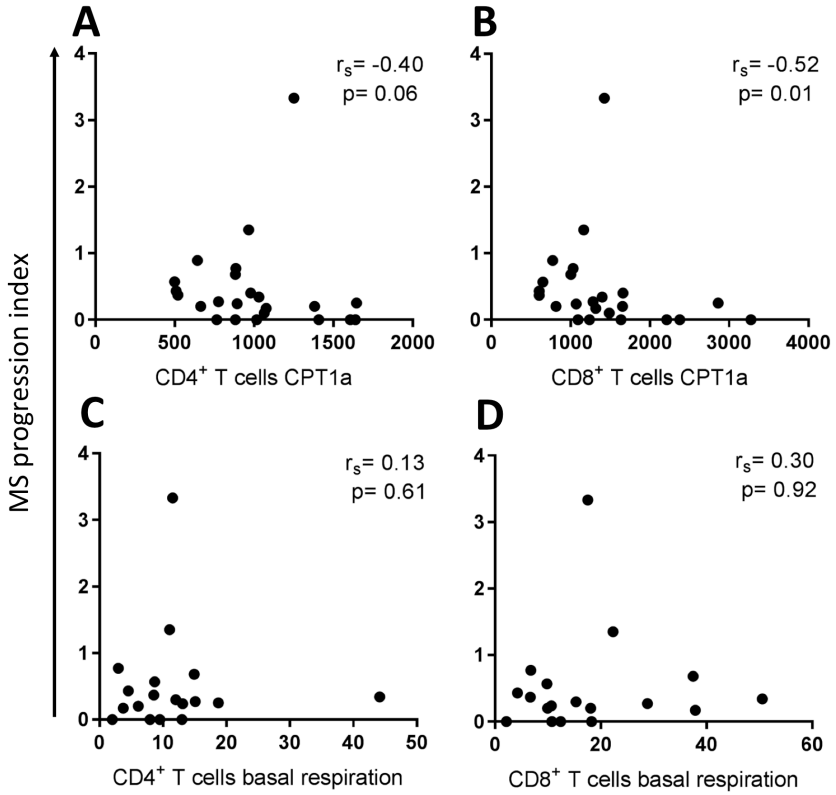

Figure 34: Correlation analyses between MS disease progression index and metabolic and flow cytometric data in $CD4^+$ and $CD8^+$ T cells. For each patient, the MS disease progression index was calculated by dividing the EDSS score by the time since clinical MS diagnosis and study visit, the disease duration. Correlation of MS disease progression index with CPT1a flow cytometry data in **(A)** $CD4^+$ and **(B)** $CD8^+$ T cells. n=23. Basal respiration data from Seahorse XF^e 96 analyzer metabolic assay analyses were correlated with the MS disease progression index in **(C)** $CD4^+$ and **(D)** $CD8^+$ T cells. n=18. r_S= Spearman correlation coefficient. EDSS: Expanded Disability Status Scale, CPT1a: Carnitine palmitoyltransferase I isoform a, HC: healthy control, MS: multiple sclerosis.

Taken together, the data shows a negative correlation between CPT1a and the disease progression index in T cell subtypes of the MS patient cohort. For metabolic analysis of basal respiration, an association between T cell subtypes and disease progression was not found.

4 Discussion

4.1 The DENIM Study Provides a Robust MS Patient and HC Participant Cohort

Providing a solid MS patient and HC participant background with respect to biological samples (PBMCs and saliva) as well as case report form data is the basis for pre-clinical analyses and utterly crucial in the evaluation and interpretation of the extensive laboratory data.

The strengths of the DENIM study include the meticulously matched RRMS patients and HC participants with respect to age (≤55 years), sex, BMI (≤30 kg/m^2) and smoking status. Physical and psychological comorbidities were excluded. These criteria are known to be associated with strong inflammatory effects and it is therefore essential to consider them in patient and HC participant analyses. Data from 62 MS patients and HC participants was analyzed providing a relatively large cohort of human clinical and corresponding pre-clinical data. Patients and participants had no significant physical or psychological comorbidities and medications other than immunomodulatory therapy. Furthermore, also critical for the extensive metabolic analyses, all patients and participants had not eaten for 12 hours prior to the blood draw and PBMCs were processed within thirty minutes. These clinical study criteria provide a strong foundation for the pre-clinical analyses and have not been presented in previous studies related to metabolism and MS research in human immune cells.

In the past three years, first studies analyzed immune cell metabolism in RRMS patients. In 2017, La Rocca and colleagues published data comparing PBMCs from RRMS patients without treatment, with INF-β1a treatment and healthy controls [120]. While the study provided critical metabolic data, the patients and control participants were not matched and not fasting for a time period prior to the study visit whole blood draw. De Riccardis and colleagues provided data on immunometabolism in a cohort of treatment-free MS patients in 2015 [121]. RRMS patients and HC participants were matched for age, but not for other parameters or analyzed in pairs. The subsequent text will discuss the specific

results of these studies with respect to the data provided in the DENIM study in more detail.

Taken together, while analyzing first human RRMS patients, data on patients matched one on one to HC participants with respect to key metabolic influences like BMI, age, sex and smoking status as well as fasting blood draw is missing. Furthermore, the studies did not consider physiological and psychological diseases, which are key influencing factors in MS disease (namely major depressive disorder) and have been shown to contribute to the inflammatory profile of immune cells.

The DENIM study considers key systemic modulators of inflammation and examines patients and HC participants in within group pairwise analyses. It is important to note again that the aim of the study was to examine patients in remission and the results obtained help to understand this phase of RRMS disease and to elucidate potential influences on relapse rates and disease progression.

4.2 Detection of Comparable Phenotyping Profiles of Major T cell Subpopulations in MS Patients and HC Participants

It is known that inflammation in disease states leads to a shift in the immune cell repertoire in human. Depending on the intruding pathogen, e.g. extra- or intracellular bacteria, viruses or worms, specific immune cell subtypes are activated and proliferation processes induced to fight the infection. In autoimmune diseases like MS, analyzing the specific immune cell phenotype plays a central role to better understand the disease and the cellular and humoral factors contributing to the activation of the immune system against self-structures. These analyses can help to allude to disease causing factors, development and progression as well as potentially hint to new therapeutic targets.

In the current study, a comprehensive PBMC immune cell phenotyping was performed with both cohorts to compare phenotype alterations in MS disease versus healthy individuals. Additionally, intracellular and cell surface molecules were analyzed to include metabolic and T cell exhaustion markers. Routine whole blood analyses showed decreased absolute numbers of lymphocytes in MS patients while relative numbers of

lymphocytes measured by flow cytometry did not show differences between both cohorts. Hence, although MS patients show decreased absolute numbers of lymphocytes, the distribution of T cell subpopulations is not altered in the MS patient cohort. Comparable levels of major $CD4^+$ T cell subpopulations including Tregs were detected in MS patients and HC participants. Solely $CD8^+$ $CCR6^+$ effector T cells showed alterations of the phenotypic make up with a decreased frequency in MS patients. Finally, memory T cell subpopulations did not show alterations between MS patients and HC participants.

In RRMS, shifts in the immune cell repertoire have been described. Studies could show increasing populations of activated $CD4^+$ T cell subpopulations as well as $CD8^+$ effector T cells in patients in active disease phases [137]–[139]. These cell populations were shown to become re-activated once they cross the BBB and infiltrate the CNS. Within the CNS, they migrate to inflammatory sites, induce the formation of lesions and with that neuronal demyelination. Therefore, immunomodulatory drugs approved for RRMS treatment are effective in preventing disease progression by targeting different mechanisms of actions of T cells and other immune cells. Briefly, the mechanisms of action include: the prevention of T cell activation and division, the inhibition of pro-inflammatory cytokine secretion, the trapping of T cells in lymph nodes and with that prevention of their migration into the CNS, inhibition of B cell function and the induction of Tregs [2],[140]–[142].

To date, it could be shown that the frequency of Tregs in PBMCs from RRMS patients does not seem to be altered, but may be accompanied by a decreased suppressive capacity [143],[144]. A study by Feger *et al.* revealed increased $CD4^+$ $CD25^+$ Treg frequencies in the CSF of MS patients with neuroinflammation and no differences of Treg frequency in MS patient's PBMCs [143]. These findings were accompanied by a reduced immunosuppressive capacity of Tregs. The authors suggest that peripheral Tregs are recruited from the blood to the CNS of MS patients with ongoing neuroinflammation. They further discuss their findings by stating that Tregs enrichment in the cerebral spinal fluid (CSF) of the CNS may not be sufficient to fight neuroinflammation. These results are accompanied by studies demonstrating functional impairments of Tregs, specifically impaired immunosuppressive capacities [145],[146]. Here, Treg quality is imperative over quantity. The studies demonstrate that the specific roles of Tregs in MS disease and the mechanisms of action of this cell population leave opportunities for more detailed cellular

signaling analyses in active and remission disease phases as well as peripheral blood and CSF analyses. Furthermore, the interaction with other immune cell populations with regard to cell activation and cytokine secretion should be considered.

Data on phenotypic alterations in $CD4^+$ T cell subpopulations indicate that it may not merely be the quantity of cells or the phenotypic make up leading to MS disease development and progression, but cell specific dysfunctions including potential alterations in immunometabolic signaling, which may contribute to auto-reactive processes observed in MS disease.

While the precise functions of $CD8^+$ effector T cell subpopulations are still the center of research, $CD8^+$ $CCR6^+$ T cells have been shown to be essential in Th17-mediated pro-inflammatory immune responses [50]. A decreased population of $CD8^+$ $CCR6^+$ T cells in PBMCs of MS patients may indicate the transmigration of these cells from the peripheral blood to potential inflammatory sites. However, the observed decrease of this cell population in the current study requires future detailed analyses in order to conclude a potential link to MS disease progression. Studies by other research groups have not described this effect in MS disease to date.

Due to the high proliferative capacities of memory T cell subsets and their potential of pro-inflammatory cytokine secretion upon a secondary antigen encounter, the influence of memory T cells on MS disease has been examined. In the current study, alterations in memory T cell subsets of MS patients could not be detected. Previous studies analyzed $CD4^+$ memory T cell phenotypes in MS patients and the effect of different immunomodulatory medications: decreases in T_{CM} cells in patients treated with INF-β or glatiramer acetate [147], increases in T_{CM} cells in patients treated with fingolimod [148], decreases of T_{CM} and T_{EM} cells in patients treated with dimethyl fumerate [149] and unchanged T_{CM} and T_{EM} cell populations in patients on natalizumab treatment [150]. The current study included patients with different kinds of immunomodulatory medications, which may be causative of the observed $CD4^+$ memory T cell subset phenotypes in the MS patient cohort. A study by Haegele *et al.* analyzed 16 RRMS patients and compared peripheral blood-derived and CSF-derived memory $CD4^+$ and $CD8^+$ T cell subsets to age and sex-matched healthy controls [151]. Patients were in active disease states or after relapse and free of immunomodulatory therapy. The authors detected increased $CD8^+$ T_{EM} T cell populations in peripheral blood of patients compared to healthy individuals and

discuss their finding by potential systemic immune activation and increased MS disease activity. Furthermore, Haegele and colleagues observed a reduction in $CD8^+$ T_{EM} T cells in the CSF of MS patients compared to the patient's corresponding peripheral blood samples. The authors state that this may be due to $CD8^+$ T_{EM} T cells infiltrating brain tissue and inducing inflammatory responses [151]. Alterations in $CD4^+$ memory T cell subsets were not described. The DENIM study analyzed peripheral blood samples from MS patients on different immunomodulatory therapies providing data on remission phase MS patient memory cell phenotype analyses compared to carefully matched HC participants. Future CSF sample analyses may provide more detailed information about alterations in memory T cell subsets in MS remission phase and their impact on CNS infiltration. However, considering the importance of immunometabolism, patient and control matching should be considered.

Taken together, the phenotypic analyses of the $CD4^+$ and $CD8^+$ T cell subpopulations showed little or no changes in the MS patient cohort compared to the HC participant cohort. Therefore, qualitative analyses may hint to potential dysfunctions in MS patient's immune cell subpopulations. The results of immunometabolic analyses of T cell subpopulations are subsequently discussed.

4.3 Decreased Immunometabolic Potential is specific to $CD4^+$ T cells from MS Patients

To date, it is commonly known that immune cells adapt their metabolic function based on their immediate requirements, however, little is known about immunometabolic effects in MS patients. Novel detection and analyses technologies developed in the past years are allowing detailed measurements of cellular metabolism and the definition of specific pathways involved within very short periods of time. These technologies help to allude to underlying cellular dysfunctions and eventually establish targets for new therapies.

In the current study, for the first time, the immunometabolic profile of sorted $CD4^+$ and $CD8^+$ T cells from RRMS patients was analyzed in real-time using the latest technology

with the Seahorse XF^e96 analyzer and compared to a meticulously matched HC participant cohort.

It could be shown that $CD4^+$ T cells from MS patients display a significantly decreased basal mitochondrial oxygen consumption rate. Parameters of mitochondrial function like maximal respiration, cellular spare respiratory capacity as well as ATP production also showed decreased levels in the MS patient cohort. Only measurements of mitochondrial proton leakage were comparable between both cohorts. These results allude to reduced mitochondrial function in $CD4^+$ T cells from MS patients. Furthermore, the analysis of glycolytic activity showed decreased levels in $CD4^+$ T cells from MS patients- hence, these cells are not compensating decreased mitochondrial activity by increasing glycolysis. Contrary to the observed effects in $CD4^+$ T cells, $CD8^+$ T cells from MS patients showed metabolic parameters of oxygen consumption as well as glycolytic activity comparable to those of the HC participant cohort. Interestingly, the observed effects in decreased immunometabolic activity are $CD4^+$ T cell specific in the RRMS patient cohort.

First studies on immune cell metabolism in MS patients have been published in the past three years. The study by La Rocca and colleagues detected decreased mitochondrial oxygen consumption rate activity as well as glycolytic activity in TCR-stimulated PBMCs from RRMS patients without treatment compared to HC controls [120]. No difference was found between HC and INF-β1a treated patients, however there were detectable differences in mitochondrial respiration between the no-treatment and INF-β1a patient groups. Limitations of the study are that mitochondrial respiration of the no-treatment cohort was analyzed in eleven patients only and compared to non-matched HC individuals. Whole PBMCs were analyzed and no distinction between different cell populations was made. Additionally, the authors did not consider sex effects, BMI, smoking status or physical or psychological comorbidities. No fasting blood draws were analyzed. Nevertheless, the study provides first crucial data on emerging differences in energy metabolism in immune cells from MS patients and hints to influences of current state of the art RRMS medication and immunometabolic function.

The current study sheds light into the mechanisms of immunometabolic dysfunctions in sorted $CD4^+$ and $CD8^+$ T cells from MS patients and allows conclusions about the mechanisms contributing to MS disease. The decrease in oxygen consumption and overall mitochondrial activity observed in $CD4^+$ T cells may be associated with impairments in the

ETC in MS patients. In their 2015 study, De Riccardis and colleagues analyzed ETC complexes and cellular respiration in $CD4^+$ T cells from twelve unmedicated RRMS patients and compared the data to eight age-matched healthy individuals [121]. The authors detected decreased protein levels of ETC complexes I and IV in RRMS patients. These findings were associated with decreased oxygen consumption rates and ATP-production levels in $CD4^+$ T cells from RRMS patients as well as increased levels of glycolysis and glycolysis-derived ATP. While the study provides a relatively low number of MS patients and HC participants, it provides crucial data on ETC impairments. Increased glycolytic activity was accompanied by increased GLUT1 protein levels as well as increased lactate production, which the authors describe as a potential hint to an energetic switch from OXPHOS to glycolysis in the observed cohort.
To the best of knowledge, detailed mitochondrial analyses of sorted $CD8^+$ T cells from MS patients have not been published to date. Complementing the observed effects of impaired mitochondrial complex function in $CD4^+$ T cells by De Riccardis and colleagues, it may be assumed that similar dysfunctions could have related effects in $CD8^+$ T cells. However, detailed analyses have to follow.

In addition to cellular analyses, serum tests contributed findings of metabolic dysfunction in MS. In 2013, Braidy and colleagues showed increased NADH levels in serum from RRMS patients compared to healthy individuals. The impairment in complex I (NADH dehydrogenase) ETC activity observed by De Riccardis [152] alludes to potential connections between this ETC complex impairment and increased NADH serum levels in patients. Taken together, these results strengthen the findings on immunometabolic dysfunctions in MS patients and should be taken into consideration when finding therapeutic targets in future investigations.

Furthermore, common MS symptoms and co-morbidities including fatigue and depression could be associated with impaired mitochondrial energy production, suggesting that dysregulated energy metabolism in immune cells may impact other organ systems, tissues and cells and with that contribute to systemic effects. Likewise, adaptations in energy metabolism in $CD4^+$ T cells may be due to potential dysregulations in other cells, tissues and organ systems. Here, the complex interaction of the central nervous system

and the immune system is most noteworthy and has comprehensively been reviewed [153]–[155].

The significantly decreased levels of ATP production in $CD4^+$ T cells in the current study are due to decreased OXPHOS activity [86]. Specific data of glycolytic ATP-production could not be generated with the applied method. However, in $CD4^+$ T cells of MS patients, the decrease in basal and maximal glycolytic activity accompanied by decreased OXPHOS leads to the assumption that the cellular energy production via both main cellular energy pathways is decreased in general and no compensatory switch from OXPHOS to glycolysis occurs. The decrease in OXPHOS and glycolytic activity could be described as an overall decreased cellular fitness of $CD4^+$ T cells from MS patients. The reduced cellular fitness can be also observed in the significantly decreased rate of non-mitochondrial respiration in $CD4^+$ T cells from MS patients. Non-mitochondrial respiration data entails the pentose phosphate pathway as well as glutaminolysis and amino acid metabolism, the later also contributing to citrate cycle energy production. Furthermore, maximal respiration and with that spare respiratory rates did not reach levels detected in HC participants, which furthermore indicates decreased fitness levels of $CD4^+$ T cells from MS patients and comprehensive impairments in cellular energy production.

Adiele *et al.* comprehensively reviewed the latest findings on immunometabolic defects related to MS disease in their 2017 review [156]. The authors systematically discuss key findings including the contributing factors of metal metabolism as well as dysfunctions in mitochondrial and oxygen metabolism on a cellular level in the blood and CNS. The research articles cited provide broad evidence that metabolic aspects have strong effects on MS disease prevention, course and progression. The biochemical and molecular defects involve comprehensive pathways and signaling cascades and with that require future studies to clarify the mechanism of metabolic dysfunctions in MS disease.

Future immunometabolic analyses will build on the ones presented in the current study and provide more data on cellular energy consumption, e.g. more detailed $CD4^+$ and $CD8^+$ T cell subtype analyses. Furthermore, longitudinal clinical study analyses of remission and relapse phases including the impact of treatment as well as disease monitoring via MRI imaging will shed light onto the interaction of the CNS and immunometabolic regulation in MS patients.

Comprehensive laboratory analyses of protein and gene expression as well as routine blood testing performed with the MS patient and HC participant samples from the DENIM study help to allude to potential causes of the observed cellular dysfunction in energy metabolism in T cells of MS patients and are subsequently discussed.

4.4 $CD4^+$ and $CD8^+$ T cell Subpopulations from MS Patients Express Increased Levels of the Mitochondrial Membrane Protein CPT1a

In this thesis, it could be shown that the mitochondrial membrane protein CPT1a was significantly increased in all $CD4^+$ T cell subpopulations (Th1, Th2, Th17, Th1/17, Tregs) and $CD8^+$ effector T cell subsets in the MS patient cohort compared to the HC participant cohort. Memory T cells of MS patients showed CPT1a protein levels comparable to the ones detected in the HC participant cohort. These findings are observed for the first time in a human MS study and hint to significant functional impairments in cellular energy metabolism and the mitochondrial membrane protein. Conventional $CD4^+$ T cells showed increased levels of CPT1a, a trend towards decreased IL7-Rα and no changes in IL2-Rα expression in MS patients. Furthermore, cellular PD-1 expression analyses did not reveal alterations in PD-1 expression in T cell subpopulations in MS patients. Therefore, the observed findings in metabolic dysfunction and CPT1a protein expression in $CD4^+$ T cell subsets were not accompanied by modified PD-1 expression levels in MS patients. Routine blood analyses indicated a trend toward decreased serum HDL levels in MS patients adding to potential impairments in fatty acid utilization. Moreover, it was analyzed whether modified gene expression complements the observed metabolic effects. *CPT1a* and *GLUT1* as well as key inflammatory genes *TNFα*, *NFκB1* and *NFκB3* did not reveal an adapted profile in $CD4^+$ and $CD8^+$ T cells in MS patients. Finally, correlation analyses of CPT1a protein abundance in $CD4^+$ T cells from MS patients revealed a negative correlation with the disease progression index. These data hint to a decrease of the detected elevated cellular CPT1a with increasing disability and MS disease duration over time.

Various metabolic alterations may be causative of the observed increase in CPT1a expression in MS patients' T cell subpopulations and the accompanied overall decrease in mitochondrial respiration in $CD4^+$ T cells. T cell subpopulations may compensate the dysfunctional protein by increasing its abundance and with that potentially accumulate a multitude of dysfunctional protein that is preventing regular mitochondrial respiration and, overall, hindering cellular signaling feedback pathways involved in CPT1a signaling. In line with the described effects of energy metabolism on immune cells, the group of Nieland showed that EAE symptoms can be alleviated completely by inhibiting CPT1a [157],[158]. The group used etomoxir to inhibit CPT1a, thus decreasing mitochondrial fatty acid respiration and reversing clinical symptoms. They describe their findings in part by analyzing CPT1a mutations and show that MS patients frequently do not have mutations in CPT1a, while healthy individuals frequently show CPT1a mutations leading to reduced or overall deleted activity of the protein [158],[159]. The group tested mice and rats and treated them with either etomoxir or INF-β as a control. The animals treated with the CPT1a inhibitor showed down-regulated inflammatory responses, ameliorated EAE symptoms as well as remyelination of CNS neurons compared to INF-β treated animals. The authors conclude that CPT1a mutations in humans may protect from developing MS. Furthermore, the results of etomoxir treatment in mice and rats might provide first insights into the benefit of targeting lipid metabolism for therapy and with that alleviating MS symptoms and progression. Interestingly, Shriver and Manchester showed a similar effect in EAE-induced mice where etomoxir treatment ameliorated disease symptoms and induced a decrease in pro-inflammatory cytokine production as well as CNS inflammation [160].

Raud and colleagues most recently published data on differing effects of the CPT1a inhibitor etomoxir on regulatory and memory T cells [161]. The authors showed that CPT1a is not required for effector and memory T cell responses as well as the suppressive function of regulatory T cells. They hint to potential CPT1a-independent off target effects of etomoxir and with that suggest additional pathways involved in mitochondrial impairments in EAE-induced mice, which require further detailed analyses. Especially the effect of etomoxir dose in varying T cell subtypes requires further analyses. Here, differing cellular CPT1a protein abundance in specific T cell subtypes may contribute to etomoxir effects and should be taken into consideration.

In addition to potential mutations and the involvement of signaling pathways, metabolic impairments in T cell subsets may also involve impairments in *CPT1a* mRNA to protein translation processes. To date, common modifications in gene to protein translation are known. They include post-transcriptional and post-translational processes or subunit protein modifications (comprehensively reviewed in [71],[162]–[164]). Increased CPT1a protein abundance may signal the inhibition of CPT1a gene transcription and protein translation. Furthermore, mitochondrial morphology modifications involving fusion and fission processes that have most recently been described in the context of immunometabolism in T cells [63] may provide explanations for the observed difference in mRNA and protein abundance of CPT1a. Buck and colleagues describe that mitochondrial fission seems essential for increased mitochondrial energy production in effector T cells [63]. A decrease in mitochondrial respiratory capacity may therefore be associated with compromised mitochondrial fission and fusion processes as well as mitochondrial protein abundance. Furthermore, impaired mitophagy processes leading to increased cellular ROS levels and damaged mitochondria as described by Pua and colleagues may influence T cell function with increased mitochondrial DNA and protein abundance [68]. However, future analyses require additional detailed detection methods like immunohistochemical and microscopy analyses to detect potential mitochondrial morphology alterations and CPT1a protein levels in MS patients T cells.

The availability of fatty acids is essential for their transport into mitochondria by CPT1a and with that energy production via OXPHOS. In this thesis, the MS patient cohort showed a trend of decreased HDL serum levels compared to the HC participant cohort. In MS disease, the contributions of metabolic pathways including lipid metabolism to disease progression have been studied. Corthals comprehensively reviewed immunometabolic alterations in MS and proposes a new framework for understanding MS disease as a dysfunctions of lipid metabolism [165]. In short, Corthals suggests a skewed homeostasis of lipid metabolism in immune cells during acute phase MS and pro-inflammatory processes leading to increased cellular oxidative stress and demyelination of CNS neurons. HDL as well as associated signaling proteins have been described as a potent anti-inflammatory agent essential in cellular protective processes and contributor of lipid metabolism in MS disease [166]–[169]. Decreased HDL may therefore indicate reduced cellular protection from pro-inflammatory signaling processes and cellular

damage. Furthermore, first studies with ketogenic diet (low in carbohydrates) and fasting have proven protective in human MS and murine EAE models [109],[170],[171]. Therefore, the contributions of diet in MS disease should be taken into consideration. The effects of glucose and lipids from diet in MS patients may present future targets to potentially assist MS immunomodulatory drugs and possibly alleviate disease progression by enhancing anti-inflammatory immunometabolic cell function. Here, the benefit of a fasting diet and autophagy processes, essential in the removal of cellular waste products and self-renewal, should be considered.

Various research groups studied the interactions between immunometabolism and pro-inflammatory signaling in T cells. The results of this thesis showed no alterations in pro-inflammatory and metabolic gene expression levels in $CD4^+$ or $CD8^+$ T cells of MS patients, which may be due to the fact that the observed patients were all in remission and in the non-active disease phase. Increased pro-inflammatory signaling has been observed in activated, proliferating and stimulated T cells and has even been shown to contribute to effector T cell subset distinguishments [172] and metabolic adaptations [173]. Furthermore, effector T cell responses and overall cellular metabolic adaptations have been shown to be regulated by AMPK signaling cascades [174] including NFκB and TNFα pathways, which are central pro-inflammatory signaling pathways involved cellular activation and TCR stimulation. Cretenet and colleagues have shown that TCR-stimulated $CD4^+$ helper and $CD8^+$ effector T cell subsets display distinct immunometabolic profiles with upregulated Glut1 expression levels [172]. This alludes to the assumption that, in addition to fatty acid metabolism, adaptations in metabolic pathways including glucose metabolism are crucial for T cell activation and proliferation. $CD4^+$ or $CD8^+$ T cells of MS patients in relapse and active disease state may therefore present altered metabolic and pro-inflammatory gene expression levels and should be considered in future analyses.

Furthermore, PD-1 receptor analyses were performed on T cell subpopulations. The results complemented the data on CPT1a protein expression in T cell subsets as well as metabolic assay analyses of $CD4^+$ and $CD8^+$ T cells. To date, to the best of knowledge, PD-1 expression on T cell subpopulations alongside of phenotypic analyses has not been analyzed in MS patients. In autoimmunity, co-inhibitory T cell receptors including PD-1 have been shown to be crucial regulators in the prevention of auto-reactivity [106] and

effective in determining cellular exhaustion in auto-immunity [104],[146],[175]. T cell exhaustion is described to include the loss of effector function, the inability to transition to quiescence or memory phenotypes, a transformed transcriptional profile as well as alterations in energy metabolic signaling [176]. Especially PD-1 receptors' direct signaling interplay with energy pathways, TCR signaling and the potential to modify cellular metabolic programs in T cells has been demonstrated [177]. Interestingly, various research groups have described immunometabolic alterations during PD-1-mediated T cell exhaustion in $CD8^+$ T cells. Here, upregulated PD-1 has been shown to drive T cell exhaustion in chronic viral infection [178], a mechanism that has been assumed to be involved in MS disease development and progression [179]. The persistent stimulation of the TCR, a mechanism of T cell exhaustion, is attenuated by PD-1 engagement leading to the inhibition of downstream pro-inflammatory signaling cascades involving PI3K and AKT. The PI3K-AKT signaling cascade is a central cellular signaling pathway involved in e.g. cellular growth, proliferation and glucose metabolism. Furthermore, Patsoukis *et al.* showed in their 2015 study that PD-1 ligation inhibits amino acid transport and metabolism as well as glycolysis, while inducing OXPHOS, FAO and upregulated CPT1a expression [177].

In the analyzed MS patient cohort of the current study, elevated CPT1a protein levels in T cell subsets and decreased OXPHOS and glycolysis levels in $CD4^+$ T cells were not accompanied by differential expression of PD-1. Moreover, mRNA expression analyses involving the PI3K-AKT signaling cascades also showed no difference in gene expression. Additional surface proteins indicating cellular exhaustions may be an additional choice for future analyses in MS patients in remission and could therefore provide more insights into potential T cell exhaustion and its interplay with metabolic signaling cascades.

Finally, it was interesting to detect a trend of decreased IL7-Rα and unaltered IL2-Rα expression in conventional $CD4^+$ T cells from MS patients in the DENIM study. Various research groups have shown, that IL-7 cytokine binding to IL7-Rα induces a negative feedback loop including the down regulation and internalization of the IL-7Rα and suppression of IL-7Rα gene expression [180]. Lawson and colleagues demonstrated in the murine EAE model that IL-7 is essential for optimal $CD4^+$ T cell activation processes including TCR stimulation and down stream signaling via STAT5 and AKT cascades [181]. The authors showed that by blocking the IL7-Rα, EAE could be prevented and ameliorated

and the expansion and activation of autoantigen-specific $CD4^+$ T cells could be inhibited. This may lead to the conclusion that low IL-7Rα levels may be beneficial in MS disease progression. Furthermore, polymorphisms have been described for the *IL7-Rα* [182] as well as the *IL2-Rα* [183] gene in MS patients. The authors show that in murine EAE models and human analyses, these genetic variants contribute to MS disease susceptibility. Gregory and colleagues discuss their findings in part by stating that the IL7-R is essential in B and T cell differentiation and therefore a potential gene target for MS development [182]. Wang and colleagues state that IL-2 is essential in T cell growth, proliferation and function and alterations in its IL2-Rα may be associated with MS disease susceptibility and progression. However, the authors furthermore discuss that previous studies on the *IL2-Rα* gene yielded conflicting results where polymorphisms showed associations with MS and others did not detect associations.

Interestingly, for RRMS, an anti-IL-2R antibody was approved by the European Medicinal Agency (EMA) in 2016 as a therapeutic drug (Daclizumab beta), which has been shown to be effective in preventing IL-2R signaling [184]–[186] and with that effector T cell activation [13]. However, extensive side effects in more than 8000 treated RRMS patients have been reported until March 2018 in Germany and the EMA withdrew the drug's authorization [187].

Taken together, past studies on *IL2-Rα* and *IL7-Rα* gene analyses have shown promising results in MS patients. The observed trend of decreased IL7-Rα expression in the current study also hints to alterations in this receptor and potential effects on MS patients in remission. Future evaluations should also consider gene expression analyses to further clarify the associations with T cell function in MS disease.

To analyze a potential association between MS disease severity and duration (progression index) with CPT1a protein expression, correlation analyses were performed. The results suggest decreasing CPT1a protein abundance in $CD4^+$ T cells with increasing disease course and severity in the MS patient cohort compared to the HC participant cohort. A study by Koffman *et al.* points out the limitations of the progression index and that it may be misleading when used for very short or very long disease durations [188]. To further analyze the associations between MS disease severity and duration and CPT1a protein levels in T cell subsets, larger cohort analyses could be considered including patients with longer courses of disease. However, the correlation results indicate alterations in CPT1a

protein abundance over time in the observed MS patient cohort compared to the HC participant cohort. To the best of knowledge, such results have not been reported to date and may add to indications of alterations in immunometabolic function in MS patients.

4.5 HPA Axis Activity Corresponds between MS Patients and HC Participants While GR and GILZ Gene Expression is Altered in $CD4^+$ and $CD8^+$ T cells

In order to detect CNS-mediated stress response mechanisms and their impact on immunometabolic function in MS patients, salivary cortisol level as well as gene expression analyses of *GR* and *GILZ* mRNA were performed. The MS patients and HC participants were free of psychological comorbidities including major depressive disorder as well as clinically significant fatigue that may impact CNS-related analyses. Similar morning and evening cortisol levels were observed in MS patients compared to HC participants, while gene expression analyses showed altered profiles in $CD4^+$ and $CD8^+$ T cells. Here, MS patients showed significantly increased *GILZ* mRNA levels in $CD4^+$ T cells and significantly decreased *GR* levels in $CD8^+$ T cells.

Alterations in salivary cortisol abundance throughout the day allude to impairments in CNS-mediated stress response mechanisms potentially affecting cellular energy metabolism and immune response mechanisms. In MS disease, the chronic activation of immune response mechanisms may impact this sensitive stress response system, or vice versa, and further deepen the understanding of the relationship of CNS and immune system interactions in MS disease onset and progression. A study by Gold *et al.* showed that salivary cortisol levels are not altered between HC participants and non-depressed MS patients [122]. The authors further analyzed MS patients with associated depressive symptoms and detected elevated evening cortisol levels compared to HC participants. A study by Powell *et al.* demonstrated elevated morning cortisol levels in RRMS patients compared to HC participants [189]. The authors further demonstrated that reported fatigue levels were associated with lower morning cortisol levels in MS patients, however, the group could not show a causal relationship between fatigue symptoms and cortisol

levels. Similar results of increased morning cortisol levels in MS patients and disease progression have been shown by Kern *et al.* [190],[191]. The results of the mentioned studies demonstrate that alterations in HPA axis activity may be associated with symptoms like fatigue and comorbidities like MS-associated depression in MS patients and not directly associated to MS disease. The DENIM study examined patients in remission, without major physical comorbidities and psychological illnesses, which may be an indicator of the observed levels of morning and evening cortisol that were comparable to the ones measured in the HC participant cohort.

While cortisol levels were comparable between both cohorts in the current study, MS patients showed alterations in *GR* and *GILZ* gene expression levels in MS patients' $CD4^+$ and $CD8^+$ T cells. One of the major targets of anti-inflammatory GILZ signaling is the inhibition of pro-inflammatory NFκB signaling. In the current study, $CD4^+$ T cells from MS patients displayed elevated GILZ levels independent of altered *GR* and *NFκB* expression, which hints to signaling mechanisms that involve additional cellular networks that should be analyzed in future studies. Additionally, GILZ has been shown to possess anti-apoptotic effects involved in the regulation of T cell activation and preventing TCR-activated apoptosis [97]. This prevention of TCR activation has also been shown to prevent NF*κB* activation and nuclear translocation to further initiate pro-inflammatory cell signaling cascades [97]. The downregulation of the GR was observed in immune cells that are preventing extracellular GC binding with respect to TCR activation (comprehensively reviewed in [192]), thereby inhibiting CNS-mediated GC influence and the induction of anti-inflammatory signaling. Furthermore, T cell cortisol response and resistance by GR downregulation has been shown to be altered in the murine EAE model. CNS infiltrates of T cells in EAE-induced mice showed increased GC resistance with downregulated GR which preceded CNS infiltration and clinical symptoms [193].

The data provided in the current study resulted from peripheral blood-derived T cells from MS patients in remission without major comorbidities and medications. The observed modifications in *GR* and *GILZ* gene expression allude to alterations in glucocorticoid signaling responses and should be analyzed further to provide potential associations with steroid treatment response in MS relapse phases.

Taken together, the analysis of CNS hormone regulation and intracellular signaling pathways are indispensable when analyzing the interaction of CNS and immunometabolic mechanisms in MS disease and frequent comorbidities like depression and fatigue.

4.6 B cells and NK cells show Modified Phenotype Profiles and CPT1a Protein Levels in MS Patients versus HC Participants

Accompanying the comprehensive T cell analyses, B cells, NK cells and monocytes were studied in order to obtain energy metabolic, phenotypic, protein level and gene expression data for the MS patient and HC participant cohorts. It could be shown, that the metabolic profile of non-$CD4^+$/$CD8^+$ T cells (mainly composed of B cells, NK cells and monocytes) from MS patients complements the one of the HC cohort with the exception of a trend toward increased basal ECAR values in MS patients. Furthermore, an increased *TNFα* mRNA expression was detected in non-$CD4^+$/$CD8^+$ T cells accompanied by a trend toward increased *CPT1a* mRNA expression. Detailed phenotypic analyses were performed to define the cellular composition of the non-$CD4^+$/$CD8^+$ T cell fraction analyzed in metabolic and gene expression assays. Here, a strikingly increased frequency of B cells and regulatory NK cells as well as decreased frequency of cytotoxic NK cells was observed in the MS patient cohort compared to the HC participant cohort. CPT1a protein levels were increased in NK cell subsets but not in B cells of MS patients. Finally, monocyte subpopulations did not show altered phenotypic data or CPT1a protein expression in MS patients.

While MS is generally considered a T cell-mediated disease and comprehensively described in this thesis, B cells have gained substantial interest in MS research. To the best of knowledge, to date, studies have not analyzed or shown potential metabolic alterations of B cells in MS patients.

In 2018 first murine studies on B cell metabolism have been published. Clarke *et al.* compared bioenergetically more active innate-like tissue resident B1 B cells to B2 B cells that are continuously produced in the bone marrow and are a distinct B cell subset from B1 B cells [194]. The authors observed metabolic adaptations including increased OXPHOS

and glycolysis as well as exogenous fatty acid dependency in B1 B cells compared to B2 B cells. Clarke and colleagues conclude that B1 B cells adapted their specific functional properties to the metabolic requirements in their tissue-resident environment. Mendoza and colleagues showed increased OXPHOS and aerobic glycolysis upon germinal center B cell response accompanied by the activation of the PI3K-AKT-mTORC1 pathway and increased gene expression involving glucose metabolism [195]. Moreover, as observed in T cells and TCR signaling, cellular fate is dependent on B cell metabolic programming and mitochondrial activation involving downstream B cell receptor signaling cascades including NFκB and mTORC1 as well as mitochondrial ROS production. This effect was demonstrated by Tsui and colleagues in their 2018 study with murine cells [196]. Impairments in B cell metabolism and activation following antigen exposure can immediately impact helper T cell fate since B cells function as APCs and helper T cells provide a secondary signal for strong B cell activation [196].

The effect of increased B cell populations in MS patients, as observed in the current study, has also been discussed by other research groups. Their role in MS has become evident by the success of α-CD20 therapy (e.g. ocrelizumab, rituximab) [197]. Baecher-Allan and colleagues discuss the beneficial effect of α-CD20 therapy by the deletion of pro-inflammatory B cell subsets that is driving the activation of T cells via antigen presentation [2]. Furthermore, they discuss the possibility of α-CD20 treatment to target mature naïve and memory B cells leaving plasma cells and immature B cells unaffected in MS patients. Interestingly, the balance of pro-inflammatory B cells secreting e.g. IL-6, TNFα, GM-CSF and anti-inflammatory B cells secreting IL-10 and IL-35 has been shown to be altered in MS patients with an increased pro-inflammatory phenotype [198]. Li and colleagues describe a GM-CSF memory B cell subset that is increased in MS patients compared to healthy individuals. Increased GM-CSF secretion by B cells from MS patients has been demonstrated to induce increased Th1 and Th17 development and with that T cell activation [198]. The authors show that upon α-CD20 therapy, the imbalance of GM-CSF secreting B cells and anti-inflammatory IL-10 secreting B cells could be reversed and address the potential of selective targeting of B cell subsets in MS disease. Hauser *et al.* showed that α-CD20 therapy correlates with decreased pro-inflammatory cytokine secretion and the absence of memory B cells in MS patients [199]. Duddy and colleagues demonstrated that the ratio of memory and naïve B cell subsets is altered in MS patients

contributing to disease activity [55]. The authors confirmed data showing that human B cell subsets display distinct cytokine profiles with anti-inflammatory IL-10 production almost exclusively observed in naïve B cells and pro-inflammatory TNFα and lymphotoxin production attributed to memory B cells. Duddy *et al.* showed that MS patients display decreased IL-10 production compared to healthy individuals.

In this thesis, T cell were the central objective and B cell subpopulations could not be further analyzed. Future analyses require more detailed experiments to promote B cell subtype distinguishments and B cell sorting for comprehensive metabolic analyses in MS patients. Here, the role of B cells contributing to T cell metabolic alterations and immunometabolic adaptations should be considered closely as well as the interplay between B and T cell subpopulations.

In addition to B cells, NK cells have also been shown to contribute to autoimmunity and to regulate and inhibit T cell survival in MS patients. In the current study, $CD56^{bright}$ $CD16^{low}$ regulatory NK cells were increased and cytotoxic $CD56^{low}$ $CD16^{bright}$ NK cells were decreased in MS patients compared to HC participants. Cytotoxic $CD56^{low}$ $CD16^{bright}$ NK cells compromise about 90% of NK cells in the peripheral blood and are found in much lower frequency in tissues compared to $CD56^{bright}$ $CD16^{low}$ regulatory NK cells [2]. Increases in $CD56^{bright}$ $CD16^{low}$ regulatory NK cells were observed to correlate with immunomodulatory and immunosuppressive treatment response in MS patients on IL2-Rα therapy (daclizumab) including reductions in brain inflammation [200]. Increased $CD56^{bright}$ $CD16^{low}$ regulatory NK cell frequencies have also been shown in MS patients on INFβ treatment [201]. While reductions in $CD56^{bright}$ $CD16^{low}$ regulatory NK cell frequency have been associated with relapse rate [202]. Furthermore, it was shown that $CD56^{bright}$ $CD16^{low}$ regulatory NK cells possess a reduced ability to inhibit the proliferation of autologous activated $CD4^+$ T cells in untreated MS patients, which the authors describe as possibly relating to dysfunctions in this NK cell subset or a reduced sensitivity of $CD4^+$ T cells from MS patients [186].

Taken together, these observations provide form data on the contributions of this innate cell type to MS disease. The elevated CPT1a levels observed in the MS cohort of the DENIM study have been described for the first time in NK cell subsets and provide primary data on potential that require further investigations.

Monocytes, as a main cellular component of the non-$CD4^+/CD8^+$ T cell fraction investigated in this study should finally be mentioned. In RRMS, monocytes have been shown to induce T and B cell responses, contribute to immunoregulation as well as pro-inflammatory cytokine secretion and CNS infiltration [203]–[205]. In the samples analyzed as part of the DENIM study, no phenotypic alterations or adapted CPT1a expression in monocyte subsets were detect in the MS patient cohort compared to the HC participant cohort. To the best of knowledge, CPT1a expression was analyzed for the first time in monocyte subpopulations in a human MS study and presents primary data on the mitochondrial membrane protein expression in these cell subsets. Conclusively, while T cell subsets and NK cells displayed altered CPT1a protein expression levels, this could not be observed in MS monocyte subtypes. These findings may allude to metabolic adaptations highly specific to immune cell subsets in MS disease and require more detailed investigations.

The gene expression analyses of the non-$CD4^+/CD8^+$ T cell fraction performed in the current study hint to pro-inflammatory activation processes associated with increased *TNFα* mRNA expression. Furthermore, slightly increased basal glycolytic rates and *CPT1a* mRNA expression were detected. However, along with monocyte populations, the non-$CD4^+/CD8^+$ T cell fraction entails B and NK cell subsets. Therefore, while this data provides first insight into immunometabolic alterations in the cell types making up the non-$CD4^+/CD8^+$ T cell fraction, succeeding metabolic analyses of sorted B cell, NK cell and monocyte subsets should be investigated in detail.

Taken together, future metabolic assays as well as protein and gene expression analyses will further deepen the understanding of immunometabolic signaling cascades in monocytes, B and NK cell subtypes and their contribution to the extensive alterations observed in T cell subpopulations in MS patients and their potential influence on disease development and progression.

Limitations, Strengths, Outlook

While the DENIM study provides a robust MS patient and carefully matched HC participant cohort, there are limitations that require mentioning. The diversity of medications taken by the MS patients did not allow valid and significant statistical analysis regarding the effect of individual types of medications on the analyzed immunometabolic functions. Furthermore, sex differences could not be statistically analyzed due to the limited MS patient and HC participant sample size. In future clinical studies the effect of medication and sex should be examined carefully whenever possible due to the strong evidence of both factors influencing MS disease susceptibility, development and progression [206]–[209]. It is important to note that the DENIM study provided comprehensive data, however, the analyses performed were limited by the availability of biomaterial, especially of sorted T cells.

The blood samples were processed following strict standard operating procedures allowing freezing and subsequent pairwise MS patient and HC participant analyses for all laboratory techniques performed. The metabolic assay was also established after comprehensive testings. However, freezing procedures have been shown to impact immune cell quality [124] and analyzing fresh samples in metabolic assays may be desirable in order to receive even higher quality readouts.

In healthy individuals, different T cell subpopulations, e.g. effector T cells, memory T cells or Tregs, have been shown to rely on distinctive metabolic programs based on their specific cellular demands and requirements. Therefore, future studies should analyze MS patients' $CD4^+$ and $CD8^+$ T cell subpopulations to narrow down specific cell subsets and observe differences in metabolic function. Considering the alterations in frequencies of B cells and NK cell subpopulations in MS patients, more detailed cell subset phenotyping of B cells, including the memory compartment, should be performed as well as comprehensive immunometabolic profile analyses in order to further investigate their effect on MS disease progression.

The study was limited by flow cytometry capacity and including additional inflammatory markers like IL-17 or IL-6 would provide further information about the pro-inflammatory characteristics of T cell subpopulations. Furthermore, serum analyses of e.g. IL-6, IFNγ, IL-

17 or TNFα as potent pro-inflammatory cytokines in MS involved in neuroinflammation and adaptive and innate immune cell activation [210] could also be completed to provide additional information about markers of systemic inflammation and associations with MS disease progression.

More comprehensive RNA analysis techniques including microarray analyses may be considered in future investigations allowing a more comprehensive exploration of genes and with that providing information about signaling cascades involving inflammatory and metabolic signaling. Here, the difference of *CPT1a* mRNA gene expression and protein abundance should be considered to help to understand the impact on immune cell function, especially in $CD4^+$ T cells. Additional protein quantification methods, e.g. SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and Western Blot analyses or broad proteomic screenings for CPT1a will add quantitative data to the observed results. Immunohistochemical microscopy analyses of CPT1a as well as staining mitochondria would also provide quantitative data on CPT1a abundance and mitochondrial morphology within T cell subpopulations. This data could allude to potential impairments in mitochondrial fusion, fission and mitophagy processes and their impact on immunometabolic function in MS patients. Additionally, sequencing of *CPT1a* may be performed complementing the results in the EAE animal model [157],[211] and potentially detecting SNPs in the *CPT1a* gene.

In upcoming clinical studies, it will be interesting to see how the inflammatory and metabolic profile changes in phases of relapses in RRMS patients and how the mitochondrial signaling pathways including the CPT1a protein may be altered during disease progression. Furthermore, the involvement of different energy pathways in immune cell subpopulations in relapses should be analyzed to broaden the understanding of the interplay between cellular energy metabolism and MS disease progression and finally lead to potential therapeutic targets. Finally, MRI imaging, as the most common biomarker for MS disease [2], could also be taken into consideration to monitor and correlate disease progression and immunometabolic parameters.

List of Abbreviations

Table 18: List of Abbreviations.

acetyl-CoA	Acetyl-coenzyme A
ADP	Adenosine Diphosphate
APC	Antigen Presenting Cell
APC	Allophycocyanin (flow cytometry)
ATP	Adenosine Triphosphate
BAI	Beck's Anxiety Inventory
BDI-II	Beck's Depression Inventory Version II
BSA	Bovine Serum Albuminum
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CPT1a	Carnitine palmitoyltransferase I isoform a
CIS	Clinically Isolated Syndrome
CRF	Case Report Form
CTQ	Childhood Trauma Questionnaire
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
DMSO	Dimethylsulfoxide
DSM-V	Diagnostic and Statistical Manual of Mental Disorders Version 5
EAE	Experimental Autoimmune Encephalomyelitis
FACS	Fluorescence Activated Cell Sorting
FAD	Flavin Adenine Dinucleotide
$FADH_2$	Flavin Adenine Dinucleotide H_2
FCC-A	Forward Scatter Area
FCS	Fetal Calf Serum
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FoxP3	Forkhead Box P3
FSMC	Fatigue Scale for Motor and Cognitive Function
FSS	Fatigue Severity Scale based on Krupp

Teff	Effector T cell
EDSS	Expanded Disability Status Scale
FACS	Fluorescently Activated Cell Sorting
GILZ	Glucocorticoid-induced Leucine Zipper
GLUT1	Glucose Transporter 1
GR	Glucocorticoid Receptor
HALEMS	Hamburger Lebensqualitätsfragebogen
HPA axis	Hypothalamic-pituitary-adrenal axis
IFNγ	Interferon γ
Ig	Immunoglobulin
IPO8	Importin 8
IL	Interleukin
LTα	Lympho toxin α
MACS	Magnetic Affinity Cell Sorting
MADRS	Montgomery-Åsberg Depression Rating Scale
MDD	Major Depressive Disorder
MFI	Median Fluorescent Intensity
MHC-I	Major Histocompatibility Complex class I
MHC-II	Major Histocompatibility Complex class II
MFSC	Multiple Sclerosis Functional Composite
M.I.N.I.	Mini International Neuropsychiatric Interview German version 5.0.0 DSM-IV
mRNA	Messenger Ribonucleic Acid
MS	Multiple Sclerosis
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide H
NK cell	Natural Killer Cell
NK T cell	Natural Killer T cell
NFκB1	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells subunit 1
NR3C1	Nuclear receptor subfamily 3 group C member 1
Oral SDMT	Oral Symbol Digit Modality Test
PBS	Phosphate Buffered Saline

PBMC	Peripheral Blood Mononuclear Cells
PE	Phycoerythrin
PPMS	Primary Progressive Multiple Sclerosis
RELA	RELA proto-oncogene, NFκB subunit
RNA	Ribonucleic Acid
RORγt	retinoic acid receptor-related orphan nuclear receptor γt
RRMS	Relapsing Remitting Multiple Sclerosis
RPMI medium	Roswell Park Memorial Institute 1640 Medium
SPMS	Secondary Progressive Multiple Sclerosis
SCC-A	Side Scatter Area
TBP	TATA Box Binding Protein
T_{CM}	Central memory T cell
TCR	T cell Receptor
T_{EM}	effector memory T cell
T_{EMRA}	terminally differentiated effector memory cells re- expressing CD45RA
Th0	T helper cell Type 1
Th1	T helper cell Type 1
Th2	T helper cell Type 2
Th17	T helper cell Type 17
Th1/17	T helper cell Type 1/17
T_N	naïve T cell
TNF	Tumor Necrosis Factor
T reg	regulatory T cell
TSC22D3	TSC22 domain family member 3

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Figure Index

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Supplements

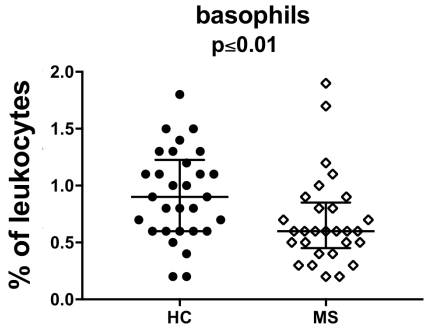


Figure 35: Within group comparisons of differential blood parameters in MS patients and HC participants. Whole blood was analyzed in a certified routine laboratory: Labor Berlin, Berlin, Germany. Blood was drawn after a 12 hour fasting period and analyzed on the same day. basophils: n=28 pairs. Wilcoxon signed-rank test with medians with interquartile ranges are displayed. , HC: healthy control, MS: multiple sclerosis.

Routine Blood Analyses

Table 19: DENIM study routine blood analysis parameters. Collected from each RRMS patient and HC participant in standard EDTA tubes. The blood analysis was performed after fasting for 12 hours.

Differential blood analysis	
Erythrocytes	Monocytes absolut
Leukocytes	Eosinophils absolut
Hemoglobin	Basophils absolut
Hematocrit	Neutrophils percent
MCV	Immature granulocytes percent
MCH	Lymphocytes percent
MCHC	Monocytes percent
RDW CV percent	Basophils percent
Thrombocytes	Immature granulocytes absolute
MPV	Eosinophils percent
Neutrophils absolut	
Lymphocytes absolut	

Clinical chemistry
LDL
HDL
CRP

Appendix

Flow Cytometry Analyses

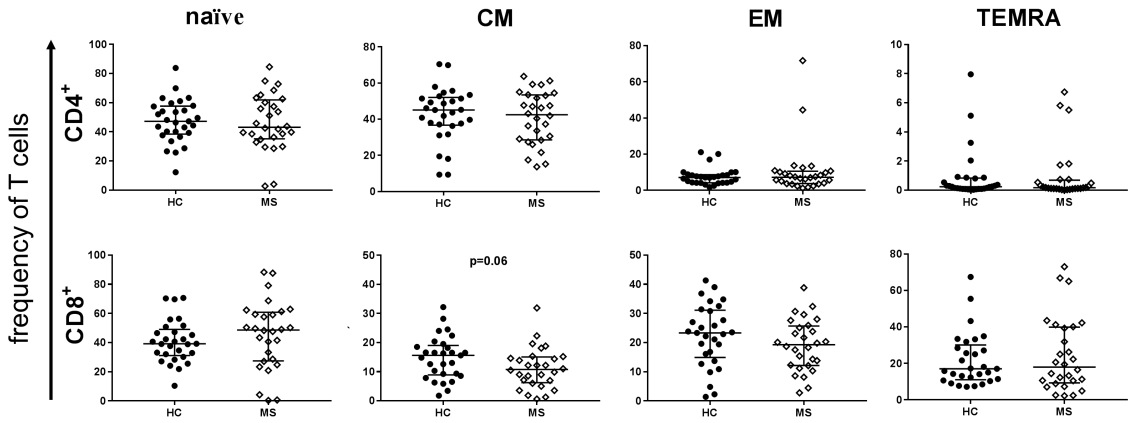


Figure 36: CD4$^+$ and CD8$^+$ memory T cell subpopulations do not show differences in frequency between MS patients and HC participants. Between group analyses of MS patients and HC participants, n=28 pairs. T cells are % of live T cells in CD3$^+$ T cell population. CD4$^+$ T cells: naïve, CM, EM, TEMRA, all p≥0.52. CD8$^+$ T cells: naïve, EM, TEMRA, all p≥0.32. CD8$^+$ CM T cells, p=0.06. Wilcoxon signed-rank test, medians with interquartile ranges are displayed. CM: central memory, EM: effector memory, TEMRA: terminally differentiated effector memory cells re- expressing CD45RA, HC: healthy control, MS: multiple sclerosis.

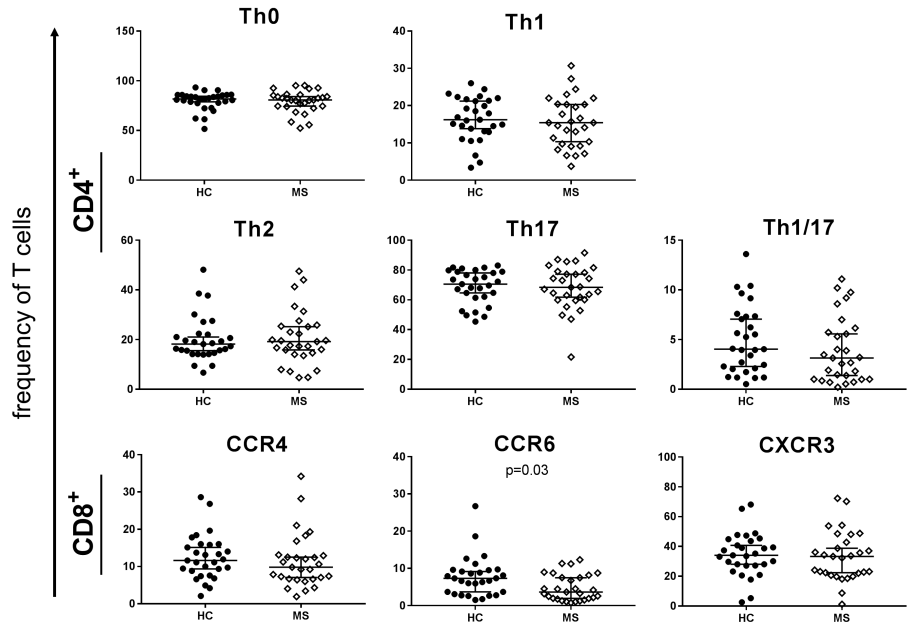


Figure 37: CD4$^+$ CD8$^+$ T cell subpopulations based on chemokine receptor expression do not show differences in frequency between MS patients and HC participants. Between group analyses of MS patients and HC participants, n=29 pairs. % of live T cells in CD3$^+$ T cell population shown. CD4$^+$ T cell subpopulation: Th0, p=0.75. Th1, p=0.64. Th2, p=0.76. Th17, p=0.92. Th1/17, p=0.62. CD8$^+$ T cell subpopulations: CCR4$^+$, p=0.31. CCR6$^+$, p=0.03. CXCR3$^+$, p=0.83. Wilcoxon signed-rank test,

medians with interquartile ranges are displayed. CCR: CC chemokine receptor, CXCR3: CXC chemokine receptor3, Th: helper T cell, HC: healthy control, MS: multiple sclerosis.

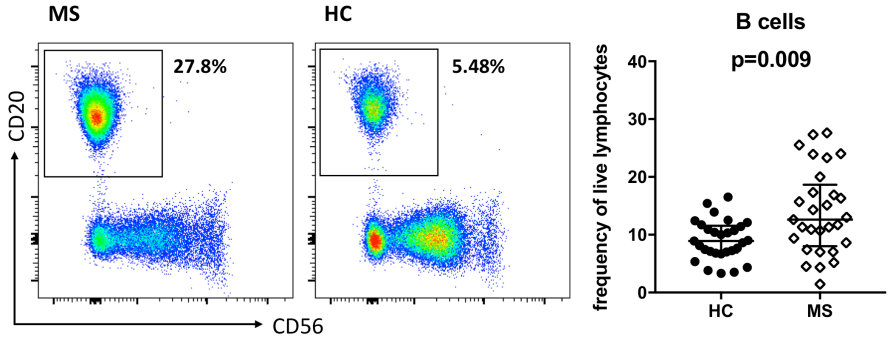


Figure 38: Increased frequency of B cells in live lymphocytes population in MS patients compared to HC participants. Between group analyses of $CD20^+$ $CD56^+$ B cells of MS patients and HC participants, n=29 pairs, p=0.0086. Displayed values in the flow cytometry blots are frequencies of $CD20^+$ cells expressed on live lymphocytes from a representative MS patient-HC participant pair. Wilcoxon signed-rank test, medians with interquartile ranges are displayed. HC: healthy control, MS: multiple sclerosis.

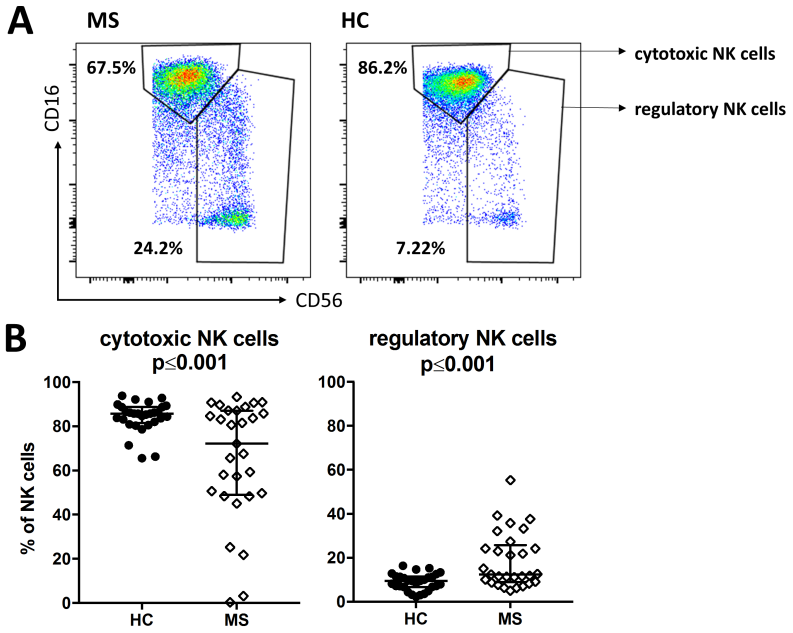


Figure 39: Frequency of cytotoxic and regulatory NK cells n NK cell population in MS patients shows significant differences compared to HC participants. Between group analyses of MS patients and HC participants, n=29 pairs. **(A)** Flow cytometry blot of $CD16^+$ $CD56^+$ cells of a representative MS patient-HC participant pair. Displayed values are frequencies of $CD56^+$ $CD16^+$ cytotoxic NK cells and $CD56^+$ $CD16^-$ regulatory NK cells expressed as a percentage of live $CD56^+$ NK cells. **(B)** Percentage of cytotoxic NK cells (p=0.0003) and regulatory NK cells (p=0.0003) between MS patients and HC participants. Wilcoxon signed-rank test, medians with interquartile ranges are displayed. NK cell: Natural Killer cell, HC: healthy control, MS: multiple sclerosis.

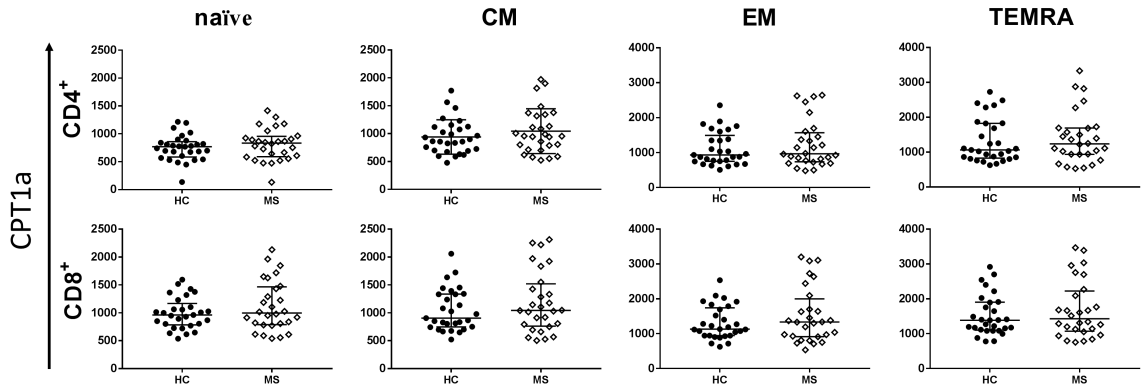


Figure 40: CD4⁺ and CD8⁺ memory subpopulations analyzed by flow cytometry for CPT1a MFI. Between group analyses for MS patients and HC participants are shown. n=28 pairs. $CD4^+$ T cell subpopulations: naïve: p=0.12. CM: p=0.15. EM: p=0.41. TEMRA: p=0.88. $CD8^+$ T cell subpopulations: naïve: p=0.18. CM: p=0.11. EM: p=0.27. TEMRA: p=0.69. Wilcoxon signed-rank test, medians with interquartile ranges are displayed. CM: central memory, EM: effector memory, TEMRA: terminally differentiated effector memory cells re- expressing CD45RA, HC: healthy control, MS: multiple sclerosis.

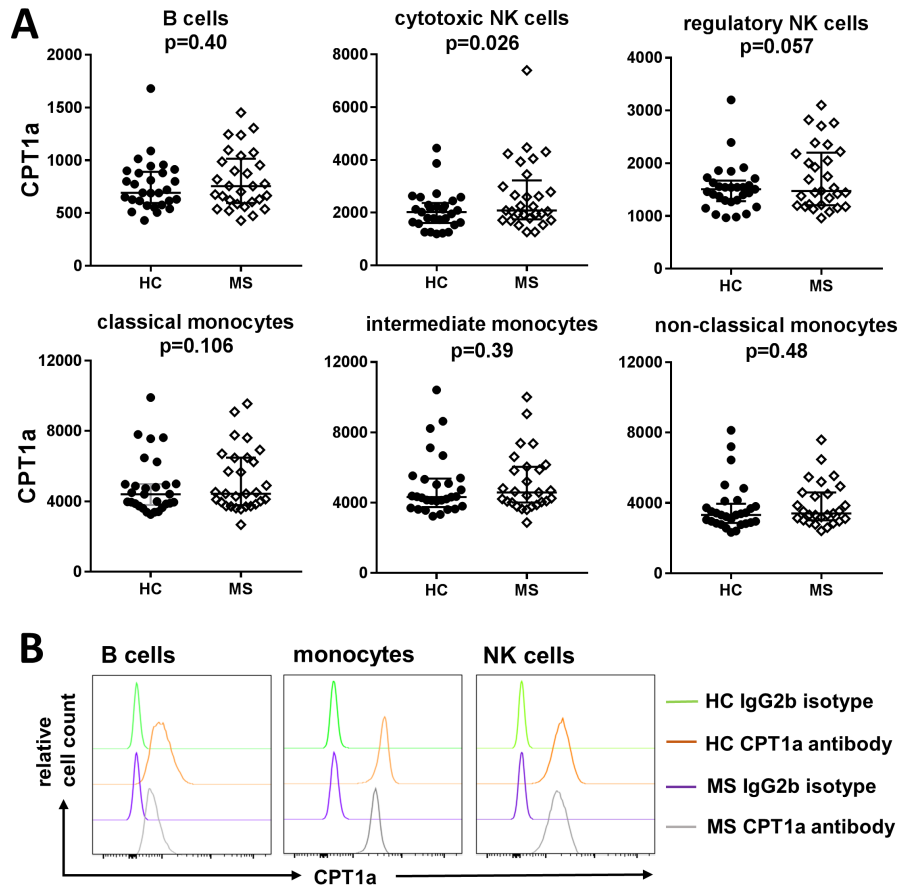
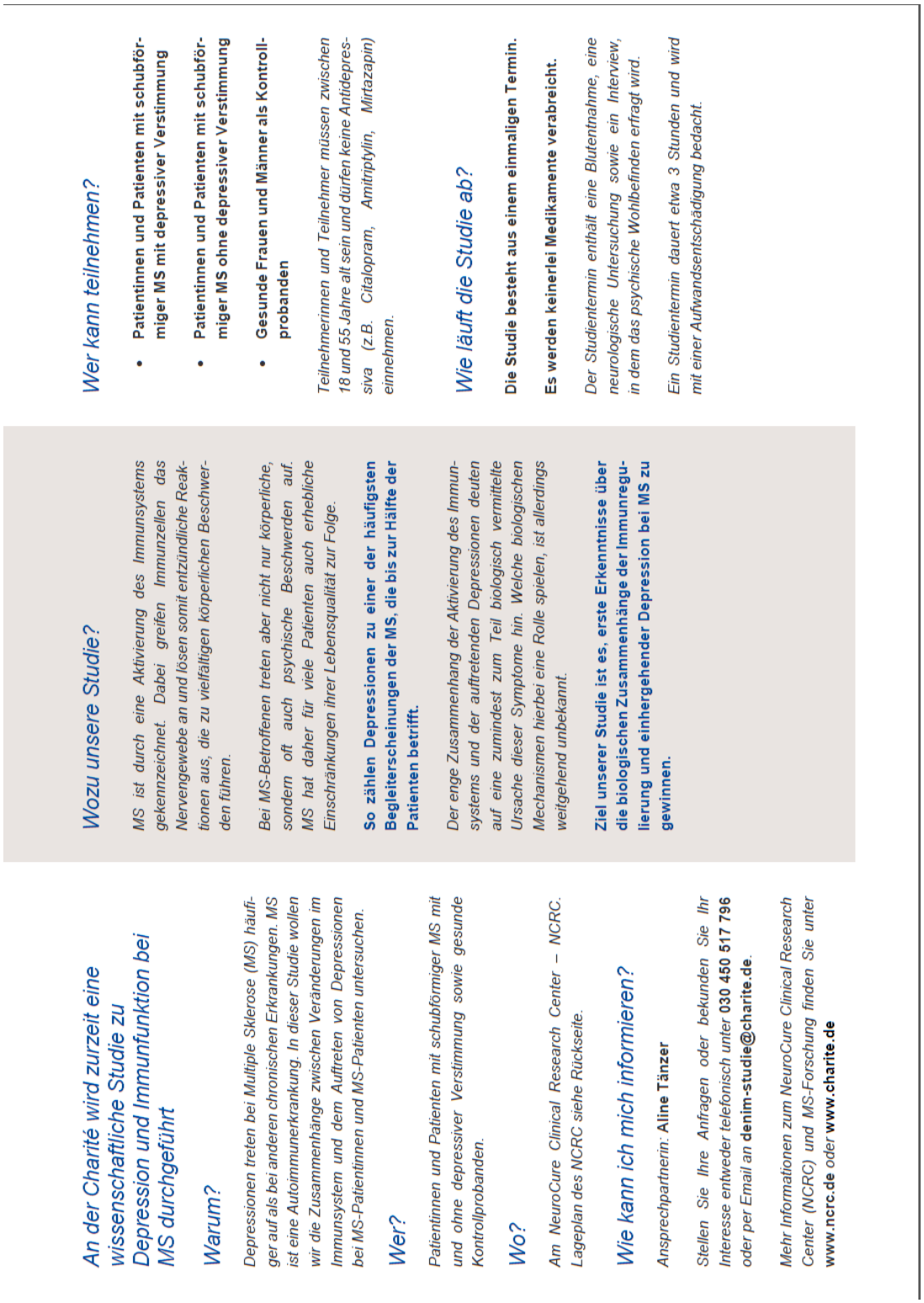


Figure 41: B cells, NK cells and monocyte subpopulations analyzed by flow cytometry for CPT1a MFI. **(A)** Between group analyses for MS patients and HC participants are shown. n=29 pairs. B cells p=0.40. cytotoxic NK cells. regulatory NK cells. $CD14^{++}$ $CD16^-$ classical monocytes. $CD14^{++}$ $CD16^+$ intermediate monocytes n=27 pairs. $CD14^+$ $CD16^{++}$ non-classical monocytes n=27 pairs. **(B)** CPT1a expression in the corresponding cell subsets of a representatice MS patients-HC participant pair. Wilcoxon signed-rank test, medians with interquartile ranges are displayed. MFI: Median Fluorescent Intensity, NK cell: Natural Killer cell, HC: healthy control, MS: multiple sclerosis.

DENIM Study Flyer

Figure 42: Depression and Immune System (DENIM) study flyer front layout and text. Prepared for information purposes for Multiple Sclerosis patient and healthy control participant recruitment.



An der Charité wird zurzeit eine wissenschaftliche Studie zu Depression und Immunfunktion bei MS durchgeführt

Warum?

Depressionen treten bei Multiple Sklerose (MS) häufiger auf als bei anderen chronischen Erkrankungen. MS ist eine Autoimmunerkrankung. In dieser Studie wollen wir die Zusammenhänge zwischen Veränderungen im Immunsystem und dem Auftreten von Depressionen bei MS-Patientinnen und MS-Patienten untersuchen.

Wer?

Patientinnen und Patienten mit schubförmiger MS mit und ohne depressiver Verstimmung sowie gesunde Kontrollprobanden.

Wo?

Am NeuroCure Clinical Research Center – NCRC. Lageplan des NCRC siehe Rückseite.

Wie kann ich mich informieren?

Ansprechpartnerin: **Aline Tänzer**

Stellen Sie Ihre Anfragen oder bekunden Sie Ihr Interesse entweder telefonisch unter **030 450 517 796** oder per Email an **denim-studie@charite.de**.

Mehr Informationen zum NeuroCure Clinical Research Center (NCRC) und MS-Forschung finden Sie unter **www.ncrc.de** oder **www.charite.de**

Wozu unsere Studie?

MS ist durch eine Aktivierung des Immunsystems gekennzeichnet. Dabei greifen Immunzellen das Nervengewebe an und lösen somit entzündliche Reaktionen aus, die zu vielfältigen körperlichen Beschwerden führen.

Bei MS-Betroffenen treten aber nicht nur körperliche, sondern oft auch psychische Beschwerden auf. MS hat daher für viele Patienten auch erhebliche Einschränkungen ihrer Lebensqualität zur Folge.

So zählen Depressionen zu einer der häufigsten Begleiterscheinungen der MS, die bis zur Hälfte der Patienten betrifft.

Der enge Zusammenhang der Aktivierung des Immunsystems und der auftretenden Depressionen deuten auf eine zumindest zum Teil biologisch vermittelte Ursache dieser Symptome hin. Welche biologischen Mechanismen hierbei eine Rolle spielen, ist allerdings weitgehend unbekannt.

Ziel unserer Studie ist es, erste Erkenntnisse über die biologischen Zusammenhänge der Immunregulierung und einhergehender Depression bei MS zu gewinnen.

Wer kann teilnehmen?

- **Patientinnen und Patienten mit schubförmiger MS mit depressiver Verstimmung**
- **Patientinnen und Patienten mit schubförmiger MS ohne depressiver Verstimmung**
- **Gesunde Frauen und Männer als Kontrollprobanden**

Teilnehmerinnen und Teilnehmer müssen zwischen 18 und 55 Jahre alt sein und dürfen keine Antidepressiva (z.B. Citalopram, Amitriptylin, Mirtazapin) einnehmen.

Wie läuft die Studie ab?

Die Studie besteht aus einem einmaligen Termin.

Es werden keinerlei Medikamente verabreicht.

Der Studientermin enthält eine Blutentnahme, eine neurologische Untersuchung sowie ein Interview, in dem das psychische Wohlbefinden erfragt wird.

Ein Studientermin dauert etwa 3 Stunden und wird mit einer Aufwandsentschädigung bedacht.

Figure 43: Depression and Immune System (DENIM) study flyer inside layout and text. Prepared for information purposes for Multiple Sclerosis patient and healthy control participant recruitment.

DENIM Study Case Report Form

The following pages include the representative full case report form (CRF) used with the RRMS patients, who were included in the Depression and Immune System (DENIM) study and went through the clinical assessment. HC participants received the same CRF excluding the MS-specific tests EDSS, MFSC and HALEMS. The MS patients each received a DENIM BMS (abbreviated for Berlin Multiple Sclerosis) and the HC participants received a DENIM BHC (abbreviated for Berlin Healthy Control) study case number. Additionally, the telephone screening forms are included, also representative for the MS patient cohort. To protect copyrights, most questionnaires or assessments are not printed here.

Telefon Screening

Study Nurse & Studienärztin/-arzt CRF

PASAT *[212]*

EDSS *[135]* ***& MSFC*** *[214]*

oraler SDMT *[134]*

Merkblatt zur Speichelabgabe

Fragebögen Studienproband/-in: BAI *[133, 216]*, ***BDI-II*** *[132, 217]*, ***FSMC*** *[220]*, ***FSS*** *[221]*, ***HALEMS*** *[222]*, ***CTQ*** *[223]*

Psychiatrische klinische Anamnese: MINI *[131]*, ***MADRS*** *[130]*

Klinische Studie:
Depression und Immunfunktion (DENIM)
BMS-Kohorte
Telefonscreening

DENIM **BMS**

Probandencode

Interviewer:_____

Datum:

⌊ ⌋ ⌊.⌋ ⌊ ⌋⌊.⌋⌊2⌋⌊0⌋⌊1⌋⌊ ⌋

Notizen

Telefonscreening

Guten Tag Frau/Herr ...

Vorstellen & Ziel der Studie	Mein Name ist … Ich bin wissenschaftliche(r) Mitarbeiter(in) in einem Forschungsprojekt an der Charité. Sie haben bereits an einer anderen Studie von uns am NCRC bei Prof. Paul teilgenommen, deswegen würden wir Ihnen sehr gerne eine weitere Studie vorstellen. In dieser Studie geht es um den Zusammenhang vom *Immunsystem und Depressionen im Rahmen von Multipler Sklerose*. Die Teilnahme ist freiwillig.
Ablauf & Entschädigung	Zunächst möchte ich Ihnen gerne einen kurzen Überblick über Ziel und Ablauf der Studie geben. Die Studie findet **einmalig** an einem Morgen/Vormittag Ihrer Wahl statt und umfasst circa 3 1/2 Stunden. Der Termin beginnt um 8.30 Uhr und umfasst eine Blutabnahme und eine neurologische Untersuchung sowie das Beantworten einiger Fragebögen. Es werden **keine Medikamente** verabreicht und Sie werden **in keiner Weise ärztlich behandelt**. Für Ihre Teilnahme erhalten Sie eine Aufwandsentschädigung in Höhe von 30 €.
Voraussetzungen	**Für unsere Studie ist es zwingend notwendig, dass Sie nüchtern erscheinen. (12 Stunden nichts gegessen)**
Pat.info & Email	Natürlich wird Ihnen alles auch noch einmal ausführlich erklärt, wenn Sie bei uns sind. **Außerdem bekommen Sie alle Informationen auch in schriftlicher Form zu Ihrem Termin.** Ihre Daten werden pseudonoymisiert gespeichert und ausgewertet.
Teilnahme?	Haben Sie soweit noch Fragen? Können Sie sich eine Teilnahme vorstellen?

1) *Patient/in kann sich eine Teilnahme an der Studie vorstellen, wünscht sich Pat.info via Email*_____

Bedenkzeit & Rückruf in zwei Wochen, Datum: ___. ___. _____ _____

2) *Patient/in nimmt an der Studie teil, weiter mit dem Telefonscreening, siehe unten:*

→ 2.) Dann würde ich Ihnen nun gerne ein paar Fragen stellen, um zu schauen, ob Sie für eine Teilnahme in Frage kommen.

Hat sich an Ihrer Adresse etwas geändert? (für ehemalige Pat. haben wir ja die Adressen im System)
Name: _____

Straße: _____

Stadt/PLZ: _____

Tel.(Beruf):_____Tel.(privat): _____

Emailadresse: _____

Alter: _____ **Geburtsdatum**:_____

Ich möchte Ihnen nun gern ein paar Fragen zu Ihrem momentanen medizinischen Zustand stellen.

Ich möchte nochmals sicher gehen, bei Ihnen liegt eine schubförmige MS Erkrankung vor, richtig? **ja ❑ nein ❑**

1. Medizinischer Zustand (momentan)

Gewicht:_____ Größe: _____ BMI: _____ (BMI= $\frac{Gewicht\ kg}{\text{Größe m2}}$; BMI ≤ 30)

Haben Sie medizinische Erkrankungen, die aktuell medikamentös behandelt werden- abgesehen von der MS? ja ❑ nein ❑

Welche?_____

_____

_____

_____

_____

Nehmen Sie derzeit MS Medikamente? Wenn ja, welche? In welcher Dosis?

_____

_____

_____

_____

_____

Falls ja: Hat sich in den letzten sechs Monaten etwas an Ihrer Medikation verändert? ja ❑ nein ❑

Falls ja: Hat sich in den letzten sechs Monaten etwas an Ihrer MS- Medikation verändert? ja ❑ nein ❑

Haben Sie in den letzten 3 Monaten Kortison bekommen? ja ❑ nein ❑

Waren Sie bereits einmal stationär in einem Krankenhaus? ja ❑ nein ❑

Warum?_____

_____

Liegt die letzte Impfung mehr als 3 Monate zurück? ja ❑ nein ❑

2. Schwangerschaft

Kann eine Schwangerschaft ausgeschlossen werden? ja ❑ nein ❑

3. Depressionen o.a. **psychiatrische Erkrankungen / in der Familie:** ja ❑ nein ❑
Wer?_____
_____
_____

3. Depressionen o.a. **psychiatrische Erkrankungen bei der Probandin/Proband:** ja ❑ nein ❑
Wie viele Episoden? _____
Zeiträume?_____
Antidepressiva?_____
_____

4. HALEMS Items 40. & 41. „Stimmung“:

40. Ich habe Lust, etwas zu tun.	1	2	3	4	5
41. Haben Sie sich in den letzten 2 Wochen an den meisten Tagen und die meiste Zeit des Tages deprimiert oder bedrückt gefühlt?	1	2	3	4	5

4. Café Frau Schneider – bitte ankreuzen und zum Termin vorbestellen!

Baguette mit 1) Räucherlachs, 2) Fenchelsalami, 3) Hirtenkäse. Obst: Apfel / Banane / Orange

WICHTIG: kein Kortison, Antidepressiva, Impfung & kein Infekt → sonst anrufen bitte!!

Entscheidung des Screenings

BESPRECHUNG IM TEAM:
PASSEND FÜR DIE STUDIE ALS PROBANDIN? ja ❑ nein ❑

Vergabe der Probandennummer: DENIM-BMS_____

Klinische Studie:
Depression und Immunfunktion (DENIM)
BMS-Kohorte
Study Nurse & Studienärztin/-arzt – CRF

Interview-Format, nicht an Patienten aushändigen!

DENIM BMS

Probandencode

Studienärztin/-arzt: _____
(BLOCKBUCHSTABEN)

Study Nurse: _____
(BLOCKBUCHSTABEN)

Datum:

⌊ ⌋⌊ ⌋.⌊ ⌋⌊ ⌋.⌊2 ⌋⌊0 ⌋⌊1 ⌋⌊ ⌋

Campus Charité Mitte – Checkliste

		Erledigt?	
1.	**Probandeninformation und Einverständniserklärung**	ja ❑	nein ❑
2.	**Study Nurse:**		
	2.1 Blutabnahme & Wiegen	ja ❑	nein ❑
	2.2 Hip Waist Ratio bestimmen	ja ❑	nein ❑
	2.3 MFSC	ja ❑	nein ❑
	2.4 SDMT orale Version	ja ❑	nein ❑
	2.5 Labor Berlin anrufen	ja ❑	nein ❑
	2.6 Forschungsblut an das Labor übergeben	ja ❑	nein ❑
	2.7 4 Salivetten: inkl. Merkblatt in den Rückumschlag	ja ❑	nein ❑
	2.8 Probandenquittung in den Rückumschlag	ja ❑	nein ❑
3.	**Studienärztin/-arzt Untersuchung und Anamnese:**		
	3.1 EDSS	ja ❑	nein ❑
	3.2 Anamnese Demografie/Medikamente	ja ❑	nein ❑
	3.3 MINI	ja ❑	nein ❑
	3.4 MADRS	ja ❑	nein ❑
	3.5 DSM-V-Specifiers	ja ❑	nein ❑
4.	**Patient/in eigenständig mit Anleitung durch Study Nurse:**		
	5.1 Fragebogen Demografie	ja ❑	nein ❑
	5.2 BAI	ja ❑	nein ❑
	5.3 BDI-II	ja ❑	nein ❑
	5.4 FSMC	ja ❑	nein ❑
	5.5 FSS	ja ❑	nein ❑
	5.6 HALEMS	ja ❑	nein ❑
	5.7 CTQ	ja ❑	nein ❑

Ort, Datum — Name Studienpersonal

Uhrzeit Blutabnahme — Start, Ende Zellisolation — Name Laborpersonal

Datum Eingang Salivetten am CBF — Speichel verarbeitet von

Einschlusskriterien

Ist die/der Patient/in zwischen 20-65 Jahre alt?	❑ ja	❑ nein → **STOP**
Besteht eine klinische gesicherte Diagnose schubförmige Multiple Sklerose?	❑ ja	❑ nein → **STOP**
Ist der BMI ≤ 30?	❑ ja	❑ nein → **STOP**
Erscheint der Patient nüchtern (Nahrungskarenz mehr als 12 Stunden) zur Studie?	❑ ja	❑ nein → **STOP**

Ausschlusskriterien

Wurde die immunmodulatorische oder immunsuppressive Therapie in den letzten sechs Monaten geändert?	❑ nein	❑ ja → **STOP**
Ist die letzte Impfung weniger als drei Monate her?	❑ nein	❑ ja → **STOP**
Bestand Kortikosteroidtherapie in den letzten 90 Tagen vor Einschluss?	❑ nein	❑ ja → **STOP**
Bestand in den letzten 8 Wochen eine infektiöse Erkrankung (Erkältung, Magen-Darm-Infekt, o.ä.)?	❑ nein	❑ ja → **STOP**
Besteht eine Schwangerschaft? Für Männer nicht zutreffend	❑ nein	❑ ja → **STOP**
Liegt oder lag eine der folgenden Erkrankungen vor?		
Epilepsie/Anfallsleiden	❑ nein	❑ ja → **STOP**
Schizophrenie/Autismus/Bipolare Erkrankungen/Demenz	❑ nein	❑ ja → **STOP**
Rheuma/Lupus/andere entzündliche Erkrankung aus dem rheumatischen Formenkreis	❑ nein	❑ ja → **STOP**
Diabetes mellitus	❑ nein	❑ ja → **STOP**
HIV/Hepatitis-Infektion	❑ nein	❑ ja → **STOP**
Koronare Herzerkrankung/Herzinfarkt	❑ nein	❑ ja → **STOP**
Schlaganfall	❑ nein	❑ ja → **STOP**
Kopfverletzung mit Krankenhausaufenthalt	❑ nein	❑ ja → **STOP**
Liegt eine Behandlung mit folgenden Medikamenten vor: Antipsychotika, Antidepressiva, Insulin, Antiepileptika	❑ nein	❑ ja → **STOP**
Gibt es klinisch-anamnestische oder paraklinische Hinweise auf eine Vorgeschichte von relevantem Medikamenten-, Drogen- und/oder Alkoholabusus im letzten Jahr?	❑ nein	❑ ja → **STOP**

Falls alle Einschlusskriterien und keine Ausschlusskriterien zutreffen,

kann der Patient in die Studie eingeschlossen werden!

Study Nurse:

Körpergröße	_____cm	**Hüftumfang**	_____cm
Gewicht	_____kg	**Taillenumfang**	_____cm
BMI	_____kg/m²	**Blutdruck**	_____ /_____ mmHg

Blutentnahme für **Forschung** durchgeführt? 10 x 10 mL Li-Heparinröhrchen 1x 1 Serumröhrchen		❑ ja ❑ nein
Blutentnahme für **Labor Berlin** durchgeführt? 1x 2 mL EDTA: kl. Blutbild+Diff., 1x 3 mL Li-Heparinplasma CRP, HDL, LDL		❑ ja ❑ nein
War der/ die Patient/ in zum Zeitpunkt der Blutentnahme **nüchtern**?		❑ ja ❑ nein
Wann hat der / die Patient/in das letzte Mal etwas gegessen?		
Uhrzeit ⌊ ⊥ ⊥⌋⌊ ⊥ ⊥ ⌋	Datum:	⌊ ⊥ ⊥⌋⌊ ⊥ ⊥⌋⌊2 ⊥0 ⊥1 ⊥ ⊥⌋

MSFC-Score	TWT (1. Strecke)	⌊ ⊥ ⊥⌋,⌊ ⊥ ⊥⌋s	
	TWT (2. Strecke)	⌊ ⊥ ⊥⌋,⌊ ⊥ ⊥⌋s	
	9 HPT dominante Hand	❑ links ❑ rechts	⌊ ⊥ ⊥⌋,⌊ ⊥ ⊥⌋s ⌊ ⊥ ⊥⌋,⌊ ⊥ ⊥⌋s
	9 HPT nicht-dominante Hand	❑ links ❑ rechts	⌊ ⊥ ⊥⌋,⌊ ⊥ ⊥⌋s ⌊ ⊥ ⊥⌋,⌊ ⊥ ⊥⌋s

PASAT Testversion A, Version 3 s ❑ **Testversion B, Version 3 s ❑**	⌊ ⊥ ⊥⌋/⌊6⊥0⌋ sek.

SDMT orale Version	⌊ ⊥ ⊥⌋/⌊9⊥0⌋ sek.

Nimmt der Patient gleichzeitig noch an anderen interventionellen Studien teil? welche:_____	❑ nein	❑ ja

Studienärztin/-arzt
1) Anamnese

Erstmanifestation der MS: Monat:_____ Jahr: _____

Datum der MS-Diagnose: Monat:_____ Jahr: _____

Gibt es Fälle von MS in der Familienanamnese? ja ❑ nein ❑

wer? _____

Schubanamnese in den letzten 12 Monaten:

Symptomatik:	Datum: *(Monat/Jahr)*	Remission?	MP i.v.?	Stationär?	
	⌊_⌋⌊_⌋.⌊_⌋ 2⌋0⌊_⌋⌊_⌋			Ja ❑	nein ❑
	⌊_⌋⌊_⌋.⌊_⌋ 2⌋0⌊_⌋⌊_⌋			Ja ❑	nein ❑
	⌊_⌋⌊_⌋.⌊_⌋ 2⌋0⌊_⌋⌊_⌋			ja ❑	nein ❑
	⌊_⌋⌊_⌋.⌊_⌋ 2⌋0⌊_⌋⌊_⌋			Ja ❑	nein ❑

Immunmodulierende Medikamente aktuell

Bitte ankreuzen, wenn zutreffend	**X**	**Seit wann?**
Copaxone 20mg		
Copaxone 40mg		
Rebif22		
Rebif44		
Avonex		
Betaferon / Extavia		
Tysabri/Natalizumab		
Gilenya/Fingolimod		
Aubagio/Teriflunomid		
Tecfidera/Fumarat		
Lemtrada		
Plegridy		
Andere		

Aktuelle Medikamente und Vitamninpräparate

Welches?	Dosis?	Seit wann?

Begleiterkrankungen

Welche?	Seit wann?

2) Neurostatus

VS	HS	PY	CB	SE	DB	CE	GS	EDSS

DENIM - Studie
PASAT
Baseline

DENIM-BMS_____ Bitte Patientencode	_____ ausfüllende Person (BLOCKBUCHSTABEN)	**Datum:** ⌊_⌋⌊_⌋.⌊_⌋⌊_⌋.⌊_⌋⌊_⌋

CHARITÉ

NeuroCure, AG Neuroimmunologie, Charité
EDSS + MSFC Verlaufsdokumentation

Bitte Patientencode aufkleben

Datum: _____

Untersucher: _____

CHARITÉ

NeuroCure, AG Neuroimmunologie, Charité
EDSS + MSFC Verlaufsdokumentation

Datum: _____

Untersucher: _____

CHARITÉ

Studie: Depression und Immunfunktion – DENIM
Kohorte: BMS

Neuro Cure Clinical Research Center

Pat. ID: DENIM-BMS: _____
Datum: _____

oraler SDMT - Symbol Digit Modalities Test - Patientenblatt

Studie: Depression und Immunfunktion – DENIM Probandennummer: DENIM-BMS_____

Merkblatt zur Speichelabgabe

Im Rahmen unserer Studie „Depression und Immunfunktion“ untersuchen wir unter anderem die täglichen Schwankungen des Hormons Kortisol im Speichel.

Deshalb bitten wir Sie an zwei **aufeinanderfolgenden** Tagen Speichelproben an den folgenden Zeitpunkten (**jeweils direkt nach dem Aufstehen und um 21.00 Uhr**) zu sammeln:

Bitte vermerken Sie in dieser Tabelle nach der Speichelabgabe
Datum und Uhrzeit der Speichelabgabe.

	Tag 1 Datum: ___.___.2016		**Tag 2** Datum: ___.___.2016	
	Direkt nach dem Aufwachen	**21.00 Uhr**	**Direkt nach dem Aufwachen**	**21.00 Uhr**
Wann genau gesammelt? (Uhrzeit)	__:__ h	__:__ h	__:__ h	__:__ h
Probenröhrchen	DENIM-BMS001 Tag 1 Aufwachen	DENIM-BMS001 Tag 1 21:00h	DENIM-BMS001 Tag 2 Aufwachen	DENIM-BMS001 Tag 2 21:00h

Beispiel der Beschriftung der Probenröhrchen zur Speichelabgabe:

DENIM-BMS001
Tag 1
21.00h

Name des Probenröhrchens DENIM-BMS001

Tag: Tag 1 oder Tag 2

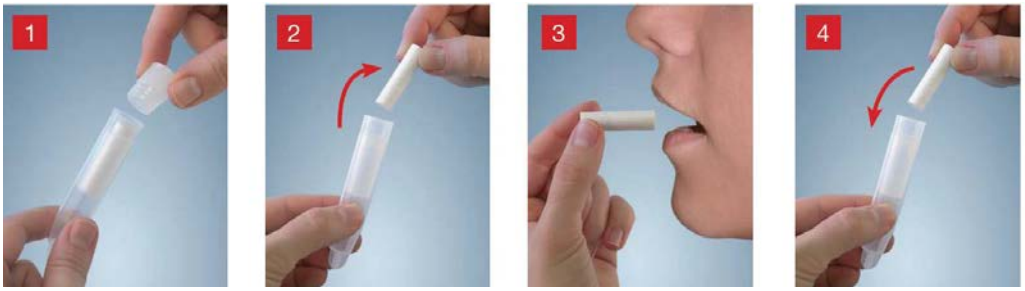
Zeit: Aufwachen oder 21.00h

Bitte benutzen Sie entsprechend der Zeitpunkte (s. oben) das richtige Röhrchen

Achten Sie dabei auf den korrekten Tag und die korrekte Uhrzeit

Studie: Depression und Immunfunktion – DENIM Probandennummer: DENIM-BMS_____

Durchführung der Speichelabgabe



1. Ziehen Sie die blaue Verschlusskappe ab.
2. Entnehmen Sie die Watterolle.
3. Legen Sie die Watterolle seitlich zwischen Kiefer und Wange in den Mund und bewegen Sie mit geschlossenem Mund für 60 Sekunden den Kiefer leicht nach rechts und links.
4. Stecken Sie die Watterolle wieder in das Röhrchen zurück, verschließen Sie das Röhrchen gut mit dem blauen Deckel und legen es zurück in den Umschlag.

Es ist wichtig die Speichelröhrchen **kühl zu lagern**, am besten im Kühlschrank!
Bitte nicht in die Sonne oder auf die Heizung legen!

Bitte beachten!!! 30 Minuten von der Speichelprobe bitte:

- Keine anstrengenden Aktivitäten
- Nicht rauchen und nicht essen
- Nicht die Zähne putzen
- Kein Kaugummi kauen
- Nichts trinken außer Wasser (keinen Kaffee, Saft, etc.)

Nachdem Sie alle 4 Proben gesammelt haben, schicken Sie Ihre Proben und dieses Merkblatt bitte im beigelegten frankierten Rückumschlag per Post an uns zurück!

Vielen Dank für Ihre Mitarbeit!

Klinische Studie:
Depression und Immunfunktion (DENIM)
BMS-Kohorte
Fragebögen – Studienproband/in

DENIM **BMS**

Probandencode

Datum:

⌊ ⌋⌊ ⌋.⌊ ⌋⌊ ⌋.⌊2 ⌋⌊0 ⌋⌊1 ⌋⌊ ⌋

Fragebogen Demografie

Bitte beantworten Sie uns hier einige Fragen zu Ihrer Person und Ihren Lebensumständen.

Alter_____

Geschlecht

- ○ männlich
- ○ weiblich

Wie ist Ihr Familienstand?

- ○ Alleinstehend
- ○ In einer festen Partnerschaft
- ○ Verheiratet/Lebensgemeinschaft
- ○ Verwitwet
- ○ Getrennt/geschieden

Wie ist Ihre Wohnform?

- ○ Allein lebend
- ○ Bei den Eltern lebend
- ○ Mit Ehe-/Lebenspartner lebend
- ○ Wohngemeinschaft
- ○ Betreutes Wohnen
- ○ Alten-/Pflegeheim
- ○ Andere: _____

Haben Sie Kinder?

Anzahl der Kinder:_____
Davon in Ihrem Haushalt lebend:_____

Was ist Ihr höchster Schulabschluss?

- ○ Keinen Schulabschluss
- ○ Hauptschulabschluss
- ○ Mittlere Reife
- ○ Fachabitur
- ○ Abitur
- ○ Hochschul-/Fachhochschulabschluss

Sind Sie derzeit erwerbstätig?

- ○ Ja, ganztags
- ○ Ja, mindestens halbtags
- ○ Ja, weniger als halbtags
- ○ Nein, in Ausbildung
- ○ Nein, arbeitslos/erwerbslos
- ○ Nein, berentet
- ○ Nein, _____

Haben Sie in Ihrem Leben jemals regelmäßig geraucht – auch wenn nur gelegentlich?

- ○ Ja
- ○ Nein

Falls ja:

Rauchen Sie zurzeit – auch wenn nur gelegentlich?

- ○ Ja
  - ○ Was rauchen Sie? (z.B. Tabak, Cannabis)

    _____
  - ○ Wie viele Zigaretten am Tag?_____
  - ○ Seit _____ Jahren
- ○ Nein

Falls nein:

Wann haben Sie in Ihrem Leben regelmäßig geraucht – auch wenn nur gelegentlich?

In welchem Zeitraum haben Sie geraucht:_____ Jahre

- ○ Was haben Sie geraucht? (z.B. Tabak, Cannabis)

  _____
- ○ Wie viele Zigaretten am Tag?_____

Für weibliche Teilnehmer:

Wann war der letzte Tag Ihrer Regelblutung?

Datum:_____

Nutzen Sie hormonelle Verhütung? („Pille“)

- ○ Nein
- ○ Wenn ja, welche? _____

  seit wann nutzen Sie das angegebene Produkt?

  Datum:_____

BAI Fragebogen

BDI-II Fragebogen

BDI-II Fragebogen

BDI-II Fragebogen

Fatigue Skala für Motorik und Kognition

Fatigue Skala für Motorik und Kognition

Fatigue Skala für Motorik und Kognition

---

vom Studienpersonal auszufüllen:

FSMC-cog =_____ FSMC-mot = _____ FSMC total = _____

Fatigue Severity Scale (FSS) nach Krupp

Lebensqualitätsfragebogen (HALEMS 10.0)

Dieser Bogen enthält viele Beschwerden, die gar nicht bei Ihnen vorliegen müssen, möglicherweise auch nie vorliegen werden. Erschrecken Sie nicht, er soll helfen, das Gespräch mit dem Arzt besser zu strukturieren. Bitte füllen sie alles aus, Danke!

1. Im Vergleich zu der Situation **vor 1 Jahr**, wie würden Sie Ihre Gesundheitssituation beschreiben?

erheblich besser	deutlich besser	besser	gleich	etwas schlechter	deutlich schlechter	massiv schlechter
1	2	3	4	5	6	7

2. Im Vergleich zur Lage **vor 4 Wochen**, wie würden Sie Ihre Gesundheitssituation beschreiben?

erheblich besser	deutlich besser	besser	gleich	etwas schlechter	deutlich schlechter	massiv schlechter
1	2	3	4	5	6	7

3. Was sind Ihre **Hauptbeschwerden**? *(Bitte markieren sie NUR die drei wichtigsten: 1., 2., 3.)*

Gehen	.....
Funktion von Armen und Händen	.....
Kribbeln, Taubheitsgefühle	.....
Schmerzen	.....
Blasenkontrolle	.....
Darmkontrolle	.....
Sehstörungen	.....
Müdigkeit	.....
Denken und Merkfähigkeit	.....
Sprechen	.....
Schlucken	.....
schlechte Stimmung	.....
Sexualität	.....

andere: .....

	gar nicht	ein wenig	mäßig	ziemlich	sehr
Wie sehr beeinträchtigen Sie diese Beschwerden?	1	2	3	4	5

*Im Folgenden finden Sie eine Liste mit Beschwerden, die bei MS-Patienten eine Rolle spielen können. Bitte markieren Sie mit einer Einkringelung wie zutreffend jede Aussage (**in den letzten 7 Tagen**) war.*
Fragen, die für Sie nicht zutreffen, bitte durchstreichen.

Missempfindungen	gar nicht	ein wenig	mäßig	ziemlich	sehr
4. Ich habe Schmerzen.	1	2	3	4	5
5. Kribbeln/Taubheitsgefühle beeinträchtigen mich.	1	2	3	4	5

Müdigkeit	gar nicht	ein wenig	mäßig	ziemlich	sehr
6. Ich muss mich tagsüber ausruhen.	1	2	3	4	5
7. Ich habe Schwierigkeiten etwas anzufangen oder zu Ende zu führen weil ich müde bin.	1	2	3	4	5
8. Körperliche Betätigung führt zu einer deutlichen Zunahme meiner Müdigkeit	1	2	3	4	5
9. Ich bin aufgrund meiner Erschöpfung oft nicht in der Lage, klar zu denken.	1	2	3	4	5

Denken	gar nicht	ein wenig	mäßig	ziemlich	sehr
10. Ich habe Schwierigkeiten, neue Dinge zu lernen.	1	2	3	4	5
11. Ich habe Schwierigkeiten, mich zu erinnern.	1	2	3	4	5
12. ich habe Schwierigkeiten, mich zu konzentrieren.	1	2	3	4	5
13. Ich habe Probleme, mehrere Dinge gleichzeitig zu machen.	1	2	3	4	5

Sehen	gar nicht	ein wenig	mäßig	ziemlich	sehr
14. Ich habe Probleme mit dem Lesen.	1	2	3	4	5
15. Ich habe Probleme mit der Orientierung oder dem Erkennen anderer Menschen.	1	2	3	4	5

16. Ich kann gehen: *(an guten Tagen)* ohne Gehhilfe mit Gehhilfe

gar nicht
bis zu 10, 20, 50,100, 200, 300, 500, 1000 m *(Zutreffendes ankringeln)*
..... km
unbegrenzt

Gehstrecke vor 1 Jahr: ……..m/km vor 2 Jahren ……m/km

Beweglichkeit / untere Extremität	gar nicht	ein wenig	mäßig	ziemlich	sehr
17. Ich habe Schwierigkeiten, Sport zu treiben oder schnell zu laufen.	1	2	3	4	5
18. Ich habe Schwierigkeiten Treppen zu steigen.	1	2	3	4	5
19. Ich habe Probleme beim Gehen innerhalb der Wohnung.	1	2	3	4	5
20. Ich habe Schwierigkeiten, sicher zu stehen.	1	2	3	4	5

Beweglichkeit / obere Extremität	gar nicht	ein wenig	mäßig	ziemlich	sehr
21. Schreiben fällt mir schwer.	1	2	3	4	5
22. Es fällt mir schwer, die Wohnung zu putzen.	1	2	3	4	5
23. Ich habe Probleme, mir eine Mahlzeit zu machen.	1	2	3	4	5
24. Ich habe Probleme beim Waschen und Anziehen.	1	2	3	4	5
25. Alleine zu essen fällt mir schwer.	1	2	3	4	5

Blase / Darm	gar nicht	ein wenig	mäßig	ziemlich	sehr
26. Ich habe Schwierigkeiten, meine Blase zu kontrollieren.	1	2	3	4	5
27. Ich habe unwillkürlichen Urinabgang.	1	2	3	4	5
28. Ich habe Schwierigkeiten, meinen Stuhlgang zu kontrollieren.	1	2	3	4	5

Kommunikation	gar nicht	ein wenig	mäßig	ziemlich	sehr
29. Ich fühle mich von meine Freunden innerlich entfernt	1	2	3	4	5
30. Ich erhalte Unterstützung von Freunden oder Nachbarn.	1	2	3	4	5
31. Ich erhalte Unterstützung von meiner Familie.	1	2	3	4	5
32. Es ist schwierig, in der Familie von meiner Krankheit zu sprechen.	1	2	3	4	5
33. Meine Krankheit beeinträchtigt den Kontakt zu anderen Menschen (Freunde, Verwandte, Familie).	1	2	3	4	5
34. Ich fühle mich ausgeschlossen.	1	2	3	4	5
35. Ich bin mit meinem Sexualleben zufrieden.	1	2	3	4	5

Stimmung	gar nicht	ein wenig	mäßig	ziemlich	sehr
36. Ich bin deprimiert über meinen Gesundheitszustand.	1	2	3	4	5
37. Meine Krankheit macht mir Angst.	1	2	3	4	5
38. Ich kann mein Leben genießen.	1	2	3	4	5
39. Ich sehe einen Sinn in meinem Leben.	1	2	3	4	5
40. Ich habe Lust, etwas zu tun.	1	2	3	4	5
41. Haben Sie sich in den letzten 2 Wochen an den meisten Tagen und die meiste Zeit des Tages deprimiert oder bedrückt gefühlt?	1	2	3	4	5
42. Haben Sie in den letzten 2 Wochen kein Interesse, gehabt irgendetwas zu tun oder haben Sie keine Freude an Dingen gehabt, die Ihnen sonst Spaß gemacht haben?	1	2	3	4	5

Gesamtbild					
43. Ich bin derzeit mit meiner Lebensqualität zufrieden.	1	2	3	4	5

44. Wie massiv beeinflusst insgesamt die MS ihre Fähigkeit ein normales Leben zu führen?
(Eine Markierung bei 1 würde bedeuten, dass die MS keinen Einfluss auf ihre Stellung im Leben, im Beruf, in der Familie hat. Eine Markierung bei 5 meint, dass die MS Sie völlig unfähig macht, ein normales Leben zu führen und damit völlig abhängig von ihrer Umwelt.)

gar nicht				**sehr**
1	2	3	4	5

CTQ

CTQ

Vielen Dank für Ihre Teilnahme, der Fragebogenteil endet an dieser Stelle.

CHARITÉ

Klinische Studie:
Depression und Immunfunktion (DENIM)
BMS-Kohorte Interviewer

Interview-Format, nicht an Patienten aushändigen!

DENIM BMS

Probandencode

Interviewer: _____

(BLOCKBUCHSTABEN)

Datum:

⌊ ⌋⌊ ⌋.⌊ ⌋⌊ ⌋.⌊2 ⌋⌊0 ⌋⌊1 ⌋⌊ ⌋

M.I.N.I.

Mini International Neuropsychiatric Interview

German Version 5.0.0

DSM-IV

Y. Lecrubier, E. Weiller, T. Hergueta, P. Amorim, L.I. Bonora, J.P. Lépine
Hôpital de la Salpétrière - Paris - FRANKREICH

D. Sheehan, J. Janavs, R. Baker, K.H. Sheehan, E. Knapp, M. Sheehan
University of South Florida - Tampa - USA

German version:
M. Ackenheil, G. Stotz, R. Dietz-Bauer, A. Vossen
Psychiatrische Universitätsklinik, München - DEUTSCHLAND

M.I.N.I. 5.0.0 **German version / DSM-IV / current (September 1999)**
Y. Lecrubier, E. Weiller, T. Hergueta, P. Amorim, L.I. Bonora, J.P. Lépine (INSERM-PARIS, FRANCE) / D. Sheehan, J. Janavs, R. Baker, K.H. Sheehan, E. Knapp, M. Sheehan (University of South Florida-TAMPA, USA)

The M.I.N.I clinical Interview entails 26 pages that were omitted here due to copyright.

MADRS

MADRS

bitte weiter explorieren:

Gibt es Fälle von Depression oder anderen psychiatrischen Erkrankungen in der Familienanamnese? ja ❑ nein ❑

wer? _____

was? _____

Besteht bei der/dem PatientIn eine depressive Erkrankung ja ❑ nein ❑

Erstmanifestation der jetzigen Episode: Monat:_____ Jahr: _____

Ist dies die Erstmanifestation der depressiven Erkrankung? ja ❑ nein ❑

Gibt es frühere Episoden der depressiven Erkrankung ja ❑ nein ❑

Anzahl früherer Episoden: _____

Wenn ja, Zeitraum früherer Episoden: _____

bitte weiter auf der nächsten Seite

Wenn eine <u>jetzige</u> depressive Episode besteht:

zusätzliche Fragen and die/den PatientIn: <u>DSM-V Specifier</u>

<u>Gemischter Subtyp</u>

An den meisten Tagen Ihrer Depression, hatten Sie da:

1. *Eine übertrieben gehobene Stimmung,*	ja □	nein □
2. *Ein erhöhtes Selbstwertgefühl,*	ja □	nein □
3. *Ein gesteigertes Redebedürfnis,*	ja □	nein □
4. *Das Gefühl, dass Ihre Gedanken rasen,*	ja □	nein □
5. *Mehr Energie und ein höheres zielgerichtetes Verhalten als sonst,*	ja □	nein □
6. *Sich in Situationen mit erhöhtem Gefahrenpotential begeben,*	ja □	nein □
7. *Ein vermindertes Schlafbedürfnis?*	ja □	nein □
Haben Freunde oder Familienangehörige eine Veränderung Ihres Verhaltens wahrgenommen?	ja □	nein □

<u>Ängstlicher Subtyp</u>

Fühlten Sie sich während der Mehrzahl der Tage Ihrer Depression

1. *Hatten Sie Angst, dass etwas Schlimmes passieren könnte,*	ja □	nein □
2. *Hatten Sie das Gefühl, die Kontrolle über sich selbst zu verlieren?*	ja □	nein □

Ende der Fragen. Bitte den Rest basierend auf vorherige Antworten ausfüllen.

3. Angespannt **(MADRS-Item 3 ≥ 4),**	ja □	nein □
4. Ruheloser als sonst (**MINI A3.c mit unruhig beantwortet)**.	ja □	nein □
5. Hatten Sie Schwierigkeiten, sich zu konzentrieren, weil Sie sich Sorgen gemacht haben (**MADRS-Item 6 ≥ 4, MINI A3.f**)	ja □	nein □

<u>Melancholischer Subtyp</u>

Diagnose melancholischer Subtyp nach **M.I.N.I (A')**	ja □	nein □

<u>Atypischer Subtypus</u>

Eine Gewichtszunahme oder Appetitsteigerung **(A3.a mit Gewichtszunahme)**	ja □	nein □
Ein gesteigertes Schlafbedürnis **(A3.b mit übermäßigem Schlafen beantwortet)**	ja □	nein □

<u>Psychotischer Subtypus</u>

Diagnose psychotischer Subtyp MADRS Item 9 ≥ 5	ja □	nein □

Diagnose basierend auf DSM-V-Specifier Depression:_____Subtypus

Acknowledgements

I would like to thank my supervisor Prof. Dr. Stefan Gold for providing the opportunity to work on this exciting topic and letting me freely discover this research project while offering invaluable support.

I want to thank Prof. Dr. Dirk Brockmann for accepting the evaluation of this thesis and for his help and guidance throughout the project.

I want to thank Prof. Dr. Friedemann Paul, Dr. Judith Bellmann-Strobl and Katharina Stößlein from the NCRC for their support and invaluable help in conducting the DENIM study. I especially want to thank Judith for contributing her experiences and providing feedback for the conceptualization of the CRF. I also want to thank all members of the NCRC for their help and involvement in the study.

My sincere gratitude belongs to all the patients and healthy control participants, who participated in this study. I want to thank the MS patients for trustfully and openly sharing their stories. For offering their free time and even taking time off from work to help us to further understand the mechanisms of MS disease.

My special appreciation goes to the members of our lab group. I want to thank Caren Ramien and Helge Hasselmann for working on the DENIM study together and contributing to its successful realization at the study site in Hamburg and the depression arm of the DENIM study. I want to thank Dr. Kostas Patas for providing guidance in setting up RNA experiments, Helge for his help in ELISA analysis and Dr. Stefanie Gamradt for her support in flow cytometry experiments. I also want to thank the HIWIs, who reliably helped in organizing this comprehensive study.

I want to thank Dr. Rainer Glauben for providing access to his lab and his continuous help and support throughout the lab work. I want to thank Inka Freise for her sincere assistance. I would also like to thank Hao Wu for our cooperative lab work with metabolic assays.

I want to thank Steffi for the proofreading of this thesis and for the memorable time in the office and lab including many captivating scientific discussions.

This thesis expresses my interest and passion for scientific research since the beginning of my University studies. I am thankful for the special people, who supported me throughout this demanding and exciting time. Above all, I am truly grateful to my friends, especially Sarah, Britta and Annett, who offered their continuous support and unconditional believe.

The National MS Society in the USA funded the research reported in this thesis.

Peer-reviewed Publications

1. **Taenzer A**, Alix-Panabières C, Wikman H, Pantel K. Circulating tumor-derived biomarkers in lung cancer. Journal of Thoracic Disease. 10.2012. 10.3978/j.issn.2072-1439.2012.08.17.

2. Hanssen A, Wagner J, Gorges T, **Taenzer A**, Uzunoglu FG, Driemel C, Stoecklein NH, Knoefel WT, Angenendt S, Hauch S, Atanackovic D, Loges S, Riethdorf S, Pantel K, Wikman H. Characterization of different CTC subpopulations in non-small cell lung cancer. Nature Scientific Reports. 06.2016. 10.1038/srep28010.

3. Ramien C, **Taenzer A**, Lupu A, Heckmann N, Engler JB, Patas K, Friese MA, Gold SM. Sex effects on inflammatory and neurodegenerative processes in multiple sclerosis. Neuroscience and Biobehavioral Reviews. 01.2016. 10.1016/j.neubiorev.2015.12.015.

4. Hasselmann H, Gamradt S, **Taenzer A**, Nowacki J, Zain R, Patas K, Ramien C, Paul F, Wingenfeld K, Piber D, Gold SM, Otte C. Pro-inflammatory monocyte phenotype and cell-specific steroid signaling alterations in unmedicated patients with major depressive disorder. Frontiers in Immunology. 11.2018. 10.3389/fimmu.2018.02693.

Eigenständigkeitserklärung

Hiermit erkläre ich, die Dissertation selbstständig und nur unter Verwendung der angegebenen Hilfen und Hilfsmittel angefertigt zu haben. Ich habe mich anderwärts nicht um einen Doktorgrad beworben und besitze keinen entsprechenden Doktorgrad. Ich erkläre, dass ich die Dissertation oder Teile davon nicht bereits bei einer anderen wissenschaftlichen Einrichtung eingereicht habe und dass sie dort weder angenommen noch abgelehnt wurde. Ich erkläre die Kenntnisnahme der dem Verfahren zugrunde liegenden Promotionsordnung der Lebenswissenschaftlichen Fakultät der Humboldt-Universität zu Berlin vom 5. März 2015. Weiterhin erkläre ich, dass keine Zusammenarbeit mit gewerblichen Promotionsbearbeiterinnen/Promotionsberatern stattgefunden hat und dass die Grundsätze der Humboldt-Universität zu Berlin zur Sicherung guter wissenschaftlicher Praxis eingehalten wurden.

Berlin, September 15th 2019 *Aline Tänzer*